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(54) Title: CELLULAR MATRIX SYSTEM AND USE THEREOF**(57) Abstract**

The invention provides a cellular matrix system which comprises cells disposed on microcarrier particles, and an in vitro defined matrix having the microcarrier particles disposed therein. The invention further provides a method for determining the effect on cellular migration/angiogenesis of a compound or effector cell of interest. The method comprises providing cells on microcarrier particles which are disposed in an in vitro defined matrix which simulates a natural environment in which the cells naturally reside; determining migration/angiogenesis of the cells into the in vitro defined matrix; including a compound or effector cell of interest in the in vitro defined matrix; redetermining migration/angiogenesis of the cells into the in vitro defined matrix which includes the compound or effector cell of interest; and determining the effect on cellular migration/angiogenesis of the compound or effector cell of interest. The invention also provides a method of promoting angiogenesis in a tissue. The method comprises selecting a tissue in which angiogenesis is desired; and introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue.

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CELLULAR MATRIX SYSTEM AND USE THEREOF

This application claims priority of U.S. Provisional
5 Patent Application No. 60/129,507, filed April 15, 1999.

This invention was made with support under NIA Grant
No. AG 101143-12. The U.S. Government may have certain
rights in this invention.

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FIELD OF THE INVENTION

The subject invention is directed generally to
cellular migration/angiogenesis, and more particularly to
a cellular matrix system comprising cells disposed on
15 microcarrier particles which are disposed within an in
vitro defined matrix, and to a method using this system
to determine the effect on cellular
migration/angiogenesis of a compound or effector cell of
interest.

20

BACKGROUND OF THE INVENTION

Throughout this application various publications are
referenced, many in parenthesis. Full citations for each
of these publications are provided at the end of the
25 Detailed Description. The disclosures of each of these
publications in their entireties are hereby incorporated
by reference in this application.

Angiogenesis, the generation of new capillaries from
pre-existing blood vessels, is critical for various
30 fundamental physiological and pathological processes such
as wound healing, tumor growth and morphogenesis (Folkman
and Shing 1992). During normal physiologic events of
morphogenesis and tissue repair, angiogenesis is tightly
regulated temporally and spatially. For example, in the
35 initial phase of cutaneous wound repair, endothelial
cells invade the fibrin/fibronectin-rich clot and form
new blood vessels. As the new wound tissue develops and

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the collagen scar forms, the blood vessels first mature and then regress (Clark 1996a). In contrast, during many pathologic processes and neoplastic diseases, new blood vessel growth proceeds unabated (Folkman 1994). Current
5 evidence indicates that the degree of tumor neovascularization correlates with metastasis and poor clinical prognosis in a variety of human cancers, including melanoma and breast carcinoma.

Neovascularization is controlled by a dynamic balance of
10 angiogenic stimulators and inhibitors. Angiogenesis requires not only up-regulation of stimulators of vessel growth, but also down-regulation of inhibitors of vessel growth (Iruela-Arispe and Dvorak 1997). Elucidating specific molecules and mechanisms that regulate
15 angiogenesis in vivo and in vitro should provide potential new therapeutic modalities to promote neovascularization in cutaneous wounds, and to inhibit angiogenesis in tumors, such as melanoma.

Angiogenesis is a complex process that involves many
20 sequential steps including adhesion, invasion, migration, proliferation and capillary tube formation (Folkman 1994; Arnold and West 1991; Bischoff 1997). The extracellular matrix (ECM) is involved in almost every step of angiogenesis, and angiogenesis occurs within the
25 interstitial ECM.

In the most commonly used assays of in vitro angiogenesis, endothelial cells are cultured on or in a fibrin gel, collagen gel or commercially available laminin gel (MATRIGEL™). Following appropriate
30 stimulation by angiogenic factors, cells aggregate and then differentiate into capillary-like structures (Blair et al. 1997; Goto et al. 1993; Madri et al. 1988; Sankar et al. 1996). Although these systems represented a major step forward in angiogenesis research, the quantification

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of capillary-like sprouts in these systems is difficult and results are often variable. Of more fundamental concern, however, is whether such in vitro systems simulate vasculogenesis rather than angiogenesis (Risau and Lemmon 1988). A promising in vitro model of sprout angiogenesis was reported by Nicosia and Ottinetti (1990). They embedded sections of rat aorta into a fibrin matrix. Over a period of several days, small tubes comprised of endothelial cells grew from the aorta muscularis (Nicosia and Ottinetti 1990). This system, however, has two major disadvantages: the tissue is not of human origin; and, the endothelial cell number and condition within the aortic sections cannot be easily defined. Nehls and Drenckhahn (1995a and 1995b) developed another system in which bovine pulmonary artery endothelial cells cultured on microcarrier beads were embedded into a bovine-derived fibrin gel to study angiogenesis. However, even in unstimulated conditions, many endothelial cells individually migrated off the microcarrier beads and invaded the fibrin gel. Nevertheless, using a similar model, Koblizek et al. (1998) demonstrated that angiopoietin-1 induced angiogenesis of rat adrenal-cortex-derived endothelial cells.

25 A need continues to exist for reliable, reproducible in vitro angiogenesis assays, which can be used to identify angiogenic stimulators and inhibitors for use in, for example, wound healing and cancer therapy, respectively.

30

SUMMARY OF THE INVENTION

The subject invention addresses this need by providing a cellular matrix system which comprises: cells of a selected cell type disposed on microcarrier

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particles; and an in vitro defined matrix having the microcarrier particles disposed therein. The in vitro matrix simulates a natural environment in which the cells naturally reside. In one particular embodiment, the
5 system is a cellular angiogenesis system comprising endothelial cells disposed on microcarrier particles and an in vitro defined fibrin matrix having the microcarrier particles disposed therein.

The invention further provides a method of
10 determining the effect on cellular migration/angiogenesis of a compound or effector cell of interest. The method comprises: providing cells of a selected cell type on microcarrier particles which are disposed in an in vitro defined matrix, wherein the in vitro matrix simulates a
15 natural environment in which the cells naturally reside; determining migration/angiogenesis of the cells into the in vitro defined matrix; including a compound or effector cell of interest in the in vitro defined matrix; redetermining migration/angiogenesis of the cells into
20 the in vitro defined matrix which includes the compound or effector cell of interest; and determining the effect on cellular migration/angiogenesis of the compound or effector cell of interest. In one particular embodiment, the method is for determining the effect on angiogenesis
25 of a compound or effector cell of interest and comprises: providing endothelial cells, preferably human microvascular endothelial cells, on microcarrier particles which are disposed in an in vitro defined fibrin matrix; determining angiogenesis of the cells into
30 the in vitro defined fibrin matrix; including a compound or effector cell of interest in the in vitro defined fibrin matrix; redetermining angiogenesis of the cells into the in vitro defined fibrin matrix which includes the compound or effector cell of interest; and

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determining the effect on angiogenesis of the compound or effector cell of interest.

In accordance with the subject invention, matrix components and culture conditions are optimized to
5 eliminate baseline cell migration. The assay is relatively easy and reproducible. The entire angiogenic process, including invasion, migration, tube formation, branching and network formation, can be recorded by time-lapse video-capture and analyzed by computer. This model
10 is a very powerful tool for investigations of human angiogenesis and can be applied to study the role of ECM receptors in angiogenic morphogenesis.

The invention also provides a method of promoting angiogenesis in a tissue. The method comprises:
15 selecting a tissue in which angiogenesis is desired; and introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

20 These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Figure 1 shows a three-dimensional human
25 angiogenesis model for assaying cell invasion, migration and tube formation of human dermal microvascular endothelial cells (HDMEC). (Step I) HDMEC are cultured on the surface of microcarrier beads to generate EC-beads. (Step II) EC-beads are embedded in fibrin gel,
30 collagen gel or fibrin/collagen gel, with or without presence of angiogenesis factor;

Figure 2 shows the formation of angiogenic sprouts and capillary tube-like structures by HDMEC in fibrin stimulated by VEGF, a) In control fibrin gel without

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addition of angiogenic factors, no significant sprout formation occurred after 48 hr. b) In the presence of VEGF (100ng/ml), HDMEC formed angiogenic sprouts, and invaded and migrated into the fibrin gel within 48 hr. c) By 7 days, VEGF-stimulated HDMEC formed branching capillary tube-like structures in the fibrin gel. 100X magnification;

Figure 3 illustrates the presence of lumen in capillary tube-like structures as demonstrated by confocal microscopic analysis. A) Phase-contrast photomicrograph of typical capillary tube-like structure formed by HDMEC in fibrin with 100 ng/ml VEGF. B) Series of contiguous 1 micron thick tangential cross sections of the capillary tube-like structure, obtained by reflective confocal microscopy with computerized imaging, initially revealed the top of the tube (0-3 microns) and then the presence of a central lumen (8-19 microns) between two walls. 400X magnification;

Figure 4 illustrates that VEGF stimulated sprout angiogenesis of HDMEC in fibrin in a concentration-dependent manner. Without the addition of VEGF, little to no sprout angiogenesis was observed in fibrin gel. The addition of increasing amounts of VEGF from 10 to 100 ng/ml resulted in a concentration-dependent increase in the number of beads with sprouts. Each data point represents the mean \pm standard deviation of triplicate determinations. The experiment shown is representative of 4 independent experiments;

Figure 5 shows the formation of capillary arcades and networks by HDMEC in fibrin with 100 ng/ml VEGF for 7 days. a) Overview of a capillary network. b) Branching capillary structures appeared to be composed of a linear array of multiple endothelial cells. Overview (c) and higher magnification (d) of a typical capillary network

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illustrate that networks formed as a result of branching and fusion of capillary tubes. Magnification: a) 40X, b) 100X, c) 40X, d) 200X;

Figure 6 shows a comparison of the angiogenic response of HDMEC to VEGF and VEGF-C at 48 hr. VEGF (open bars) and VEGF-C (shaded bars) both stimulated sprout angiogenesis of HDMEC in fibrin in a concentration-dependent manner. However, the angiogenic effect of VEGF-C was markedly less than that of VEGF. Each bar represents the mean \pm standard deviation of triplicate determinations. The experiment shown is representative of 3 independent experiments;

Figure 7 shows a comparison of the angiogenic response of HDMEC to bFGF and PDGF-BB at 48 hr. bFGF (open bars) stimulated sprout angiogenesis of HDMEC in fibrin with maximal response at 30 ng/ml. The angiogenic effect declined at higher concentrations of bFGF. PDGF-BB (shaded bars) in concentrations ranging from 10 to 100 ng/ml did not induce significant sprout angiogenesis of HDMEC in fibrin. Each bar represents the mean \pm standard deviation of triplicate determinations. The experiment shown is representative of 3 independent experiments;

Figure 8 shows that angiogenic blood vessels in early granulation tissue mature and then regress as wound repair progresses. Porcine wounds at 5 days (a,b), 7 days (c,d), and 10 days (e,f), were stained with Masson trichrome (a,c,e) and antibody to laminin (b,d,f). At 5 days (a,b), the wound space is almost filled with granulation tissue (gt), rich in newly forming microvessels (b), and the neoepidermis (e arrow) is migrating to close the wound. At 7 days (c,d) the neoepidermis has completely formed, the granulation tissue has organized, and the neovessels have matured and assumed a vertical orientation (d). At 10 days (e,f)

wound contraction is underway (cc) and blood vessel regression is apparent (f). The bright lines near the top of panels d and f represent laminin in the epidermal basement membrane. The brightly staining annular and
5 oval ring-like structures in panels (b,d,f) represent blood vessels. e arrow: migrating neoepidermis; gt: granulation tissue; cc: contracting collagen neomatrix. Bar = 1 mm in a,c,e; 0.25 mm in b,d,f;

Figure 9 shows that as angiogenic blood vessels
10 mature and then regress, collagen replaces fibrin in the extracellular matrix milieu of the wound. Granulation tissue at 5 days (A,D), 7 days (B,E), and 10 days (C,F) was stained with antibody to laminin (A,B,C) or with Masson trichrome (D,E,F). Note the gradually increasing
15 deposition of collagen (fibrillar material in D,E,F) as angiogenic blood vessels (A) mature and assume a vertical orientation (B) and then regress (C). Bar = 80 microns in A,B,C; 50 microns in D,E,F;

Figure 10 shows that fibrin gels (a,c) and collagen
20 gels (b,d) differentially regulated HDMEC response to VEGF at 48 hr. Without the addition of angiogenesis factors, little or no sprout formation, invasion or migration of HDMEC occurred either in fibrin gel (a) or in collagen gel (b). In the presence of VEGF (100 ng/ml)
25 HDMEC formed capillary sprouts which invaded and migrated into fibrin gel (c). In contrast, in collagen gel in the presence of VEGF (100ng/ml) HDMEC invaded and migrated into the gel individually without forming capillary sprouts (d). 200X magnification; and

30 Figure 11 shows that fibrin facilitated sprout angiogenesis of HDMEC in collagen induced by VEGF at 72 hr. VEGF (100ng/ml) stimulated HDMEC to invade and migrate into pure collagen gel individually without forming capillary sprouts (a). With the addition of 17%

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fibrin fibrils, by weight (b) or 29% fibrin fibrils, by weight (c) to collagen gel in the presence of VEGF, sprout angiogenesis occurred together with individual cell invasion and migration. VEGF induced sprout
5 angiogenesis of HDMEC in pure fibrin gel (d). 100X magnification.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides a cellular matrix
10 system which comprises: cells of a selected cell type disposed on microcarrier particles; and an in vitro defined matrix having the microcarrier particles disposed therein. The in vitro matrix simulates a natural environment in which the cells naturally reside. In one
15 particular embodiment, the system is a cellular angiogenesis system and comprises: endothelial cells disposed on microcarrier particles; and an in vitro defined fibrin matrix having the microcarrier particles disposed therein.

20 The invention further provides a method of determining the effect on cellular migration/angiogenesis of a compound or effector cell of interest. The method comprises: providing cells of a selected cell type on microcarrier particles which are disposed in an in vitro
25 defined matrix, wherein the in vitro defined matrix simulates a natural environment in which the cells naturally reside; determining migration/angiogenesis of the cells into the in vitro defined matrix; including a compound or effector cell of interest in the in vitro
30 defined matrix; redetermining migration/angiogenesis of the cells into the in vitro defined matrix which includes the compound or effector cell of interest; and determining the effect on cellular migration/angiogenesis of the compound or effector cell of interest. In one

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particular embodiment, the method is for determining the effect on angiogenesis of a compound or effector cell of interest and comprises: providing endothelial cells on microcarrier particles which are disposed in an in vitro defined fibrin matrix; determining angiogenesis of the cells into the in vitro defined fibrin matrix; including a compound or effector cell of interest in the in vitro defined fibrin matrix; redetermining angiogenesis of the cells into the in vitro defined fibrin matrix which includes the compound or effector cell of interest; and determining the effect on angiogenesis of the compound or effector cell of interest.

The invention also provides a method of promoting angiogenesis in a tissue. The method comprises: selecting a tissue in which angiogenesis is desired; and introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue.

This general understanding of the subject invention is described in further detail below.

As used herein, cellular migration refers to the movement of cell(s) from point A to point B. For example, in the process of wound healing fibroblasts and endothelial cells migrate from a collagenous stroma extracellular matrix into a fibrin extracellular matrix. As another example, tumor cells migrate within a host, for example, from a collagen matrix into a basement membrane extracellular matrix or from a basement membrane extracellular matrix into a collagen extracellular matrix. Embryonic and fetal cells also migrate during tissue morphogenesis from one matrix to another. Cell migrate through a basal lamina, as when white blood cells migrate across the vascular basal lamina into tissues in response to infection or injury, or when cancer cells migrate from their site of origin to distant organs via

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the bloodstream or lymphatic vessels, a process known as metastasis.

Cellular angiogenesis refers to the complex process of new blood vessel formation by endothelial cells that involves at least five steps: cell invasion and migration, and capillary-tube formation, branching and fusion to form networks. Junctions, branches, and fusions define tube extensions. A junction is defined as the location where one tube enters and two or more tubes exit. A branch is defined as a tube that has left a junction point but has not fused with another tube. A fusion is defined as a tube, either before or after a junction, that has fused with an adjacent tube.

A cellular matrix system refers to a system which can be used to study cellular migration or cellular angiogenesis.

The cellular matrix system of the subject invention comprises cells of a selected cell type disposed on microcarrier particles. A selected cell type refers to any cell type of interest for determination of cellular migration/angiogenesis properties of that selected cell type. For example, the angiogenesis properties of endothelial cells are of interest (such as cultured human dermal microvascular endothelial cells), or the migration properties of tumor cells are of interest.

The cells of a selected cell type are disposed on microcarrier particles. As microcarriers there may be used all microcarriers which consist of a biocompatible material (such as, e.g., collagen, fibrin, polymers from hyaluronic acid or hyaluronan esters (e.g. ethyl or benzyl esters) (Andreassi et al. 1991; Myers et al. 1995) (e.g. HYAFF-11™ from FIDIA Advanced Biopolymers, Abano T., Italy), fibrin binding polypeptides, dextran, gelatin, silicone, etc.) or of a suitable mixture of

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several biocompatible materials. There may be used, for example, Cytodex3® (crosslinked dextran matrix with a denatured collagen layer, 100-230 μm) (Pharmacia AB, Sweden) based on dextran Cellgen (Koken Co., Japan), a
5 reconstituted and crosslinked collagen from bovine skin, 200-500 μm , or Cultispher-G® (macroporous gelatin microcarrier) (Hyclone, Greiner, Germany) based on gelatin.

The size of the microcarriers is not critical.
10 Usually, however, the carriers have a diameter of 50 to 500 μm . Microcarriers of a diameter below 50 μm are so small that their surface is generally not sufficient for cell growth. Microcarriers of a size higher than 500 μm are so large that a high coverage with cells is generally
15 difficult to achieve. Furthermore, these particles are not optimal for transplantation into in vitro assays or tissue.

The microcarriers can be pre-coated in order to improve the adhesion of the cells and enhance the extent
20 of coverage of the microcarriers. For the coating, proteins of the extracellular matrix, such as fibronectin or collagen, or whole eukaryotic cells (e.g. fibroblasts incapable of proliferation) or components thereof (e.g. membrane fractions) may be used.

25 The microcarrier particles are disposed in an in vitro defined matrix. The matrix simulates a natural environment in which the selected cell type naturally resides. For example, in wound repair the cell may be a skin fibroblast or endothelial cell and its natural
30 environment is collagenous stroma. The skin fibroblast or endothelial cell, during wound repair, naturally migrates from the collagenous stroma into a fibrin clot which fills the wound. The in vitro matrix of the subject invention is chosen to simulate the collagenous

stroma within which the skin fibroblasts or endothelial cells naturally reside. For example, the in vitro matrix can be a contracted collagen gel produced according to the methods described in U.S. Patent No. 5,935,850, 5 issued August 10, 1999, the contents of which are incorporated herein by reference.

As used herein, a matrix is an interdigitating polymer bundle which refers to a scaffold in the cell's external environment with which the cells may interact 10 via specific cell surface receptors. Such a matrix can be a naturally occurring extracellular matrix such as, for example, collagen, fibrin, fibronectin, or hyaluronic acid, or an artificial extracellular matrix. Artificial extracellular matrices are biocompatible and include, for 15 example, dextran polymers, polyvinyl chlorides, polyglycolic acids, polylactic acids, polylactic coglycolic acids, and silicon. A description of artificial (or synthetic) extracellular matrices is provided in Putnam and Mooney (1996).

20 In order to be useful for the study of the effects of a compound or effector cell of interest on cellular migration/angiogenesis, the in vitro matrix is "defined". As used herein, an in vitro "defined" matrix refers to a matrix having defined, i.e. known, components. For 25 example, purified fibrin gels substantially free of stimulators or inhibitors are "defined" matrices, as are such fibrin gels with precise amounts of other plasma components added thereto. A fibrin gel with such other components added thereto is encompassed by the term 30 "fibrin" matrix as used herein.

As also used herein, a cellular migration/angiogenesis stimulator is a compound or effector cell which stimulates cells of the selected cell type to migrate in or from the in vitro defined matrix (a

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cellular migration stimulator), or which stimulates cells of the selected cell type to form new blood vessels (angiogenesis) (a cellular angiogenesis stimulator). Stimulates refers to a statistically significant increase
5 in migration or angiogenesis. A cellular migration/angiogenesis inhibitor is a compound or effector cell which inhibits cells of the selected cell type from migrating in or from the in vitro defined matrix (a cellular migration inhibitor), or which
10 inhibits cells of the selected cell type from forming new blood vessels (angiogenesis) (a cellular angiogenesis inhibitor). Inhibits refers to a statistically significant decrease in migration or angiogenesis. Substantially free means that any stimulators or
15 inhibitors that may be present are present in such an amount that negligible or statistically insignificant increases or decreases in the amount of migration/angiogenesis occur in the presence of such an amount of the stimulators or inhibitors.

20 In one embodiment of the subject invention especially useful for the study of angiogenesis, the cells are cultured human dermal microvascular endothelial cells disposed on microcarrier beads in a human derived defined fibrin matrix.

25 In alternate embodiments where one wishes to examine the cellular migration/angiogenesis of the cells of the selected cell type through the in vitro defined matrix toward another cell type, the cellular migration/angiogenesis system can further comprise cells
30 of a different selected cell type overlaid by or admixed with the in vitro defined matrix. "Different" refers to cells of a type distinguishable from the cells of the selected cell type (or detectable separate from). For example, one may wish to examine the formation of new

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blood vessels by endothelial cells induced by tumor cells. In one embodiment, this can be done by providing a layer or aggregate of tumor cells and having the in vitro defined matrix overlying the layer or aggregate of
5 cells (the in vitro defined matrix has the microcarrier particles disposed therein which have the endothelial cells disposed thereon). In another embodiment, this can be done by providing a droplet of tumor cells and having the in vitro defined matrix overlying the droplet (the
10 droplet may be an agarose droplet, for example, or may be the same material as the in vitro defined matrix or a different defined matrix).

Having thus described the cellular matrix system according to the subject invention, the system can be
15 used to determine the effect on cellular migration/angiogenesis of a compound or effector cell of interest. A compound of interest refers to a biological response modifier. Examples of such biological response modifiers include, but are not limited to, chemotactic
20 factors, growth factors, cytokines, autocoids, and prostanoids. An effector cell of interest refers to a cell that causes an effect of interest.

If the in vitro defined matrix overlies cells of a different selected cell type (such as in the embodiment
25 described above where one wishes to examine the formation of new blood vessels by endothelial cells toward or induced by tumor cells), then one would detect the migration/angiogenesis of the cells of the selected cell type toward the cells of the different selected cell
30 type.

It should be readily apparent to those skilled in the art that the migration/angiogenesis of cells can only be monitored or studied if the cells are detectable. This can be accomplished in several ways. In one

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embodiment the cells may be visualized with a light microscope. Alternatively, the cells can be labeled with a detectable marker. Such detectable markers are known in the art, and include, for example, radioactive labels, 5 fluorescent labels, vital dyes (these non-toxic dyes stain living cells), and labels added by molecular manipulation (such as the β gal gene). The migration/angiogenesis of the labeled cells can thus be monitored.

10 Cells of a selected cell type are disposed on microcarrier particles using methods known in the art (one such method is further detailed below). These "coated" microcarrier particles are then disposed in the in vitro defined matrix also using methods known in the 15 art (one such method is further detailed below).

A fibrin matrix which is substantially free of fibronectin as well as stimulators and inhibitors of cell migration/angiogenesis can be made in accordance with U.S. Patent Application No. 09/500,512, filed February 9, 20 2000, the contents of which are incorporated herein by reference. This method is further detailed in the Materials and Methods section below.

A compound of interest can be introduced into the in vitro defined matrix using methods known in the art (one 25 such method is further detailed below).

This entire cellular matrix system and method can be automated. In such an automated system, cultured endothelial cells are transferred to microcarrier beads robotically. Appropriate concentrations of fibrinogen or 30 other gelable material (as the in vitro matrix) are added to multiwell plates robotically. The endothelial cells on beads at a desired density are transferred to the wells robotically. The multiwell plates are then robotically handled in a manner to allow gelation of the

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in vitro matrix. Medium containing stimulatory or inhibitory factors to be tested is added to the plates robotically. Plates are incubated in the desired environment for the desired time robotically. Using a
5 microscope with a motorized stage and that is coupled to a camera or video (that is interfaced with a computer that is configured with image digitizer software), images of the beads within each well are identified, recorded and transferred to a file robotically. Alternatively,
10 plates are processed robotically to fix and stain endothelial cells migrating out from the beads. Using the same imaging system, cells in each well are identified, recorded and transferred to a file robotically. Using image analyzing software the number
15 of cell processes or tubes extending from the beads is quantified and the extension length determined.

The subject invention also provides a method of promoting angiogenesis in a tissue. The method comprises selecting a tissue in which angiogenesis is desired, and
20 introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue. This method is described more fully below. The cultured endothelial cells can be provided as an endothelial cell aggregate or as endothelial cells disposed on microcarrier particles.
25 As used herein, a tissue could be an in vitro tissue (for example, for research purposes, or for transplantation or implantation or grafting or other use in vivo), or an in vivo tissue (for example, for treatment purposes). The tissue can be naturally occurring tissue or an artificial
30 tissue. A tissue sample of particular interest would be wound tissue (such as artificial tissue for wounds). As used herein, a wound refers to both acute and chronic dermal wounds including, for example, surgical incisional wounds, traumatic wounds, cancer extirpations, radiation

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wounds, venous leg ulcers, diabetic ulcers, pressure ulcers, and burn wounds.

MATERIALS AND METHODS

5 Materials

Gelatin-coated microcarrier beads (Cytodex-3) were purchased from Pharmacia (Uppsala, Sweden). Type 1 collagen was obtained from Collagen Biomaterials (Palo Alto, CA). Dimethyl dichlorosilane, aprotinin, dibutyryl cyclic AMP, hydrocortisone, trypsin, soybean trypsin inhibitor, and EDTA, were obtained from Sigma Chemical Co. (St. Louis, MO). Endothelial cell basal medium (EBM), bovine brain extract, and epidermal growth factor were obtained from Clonetics Corp. (San Diego, CA). Normal human serum was obtained from BioWhittaker, Inc. (Walkersville, MD). Vascular endothelial cell growth factor (VEGF) was purchased from Peprotech (Rocky Hill, NJ). Basic fibroblast growth factor (bFGF) was obtained from Scios Nova, Inc. (Mountainview, CA). Platelet derived growth factor-BB (PDGF-BB) was kindly provided by Charles Hart (Zymogenetics, Seattle, WA). VEGF-C was kindly provided by Kari Alitalo (Cao et al. 1998). Human thrombin was obtained from Calbiochem (San Diego, CA).

Culture and characterization of HDMEC

HDMEC were isolated from human neonatal foreskin. Briefly, after initial harvest from minced trypsinized human foreskins, microvascular endothelial cells were further purified on a Percoll density gradient. HDMEC were cultured on collagen type 1 coated tissue culture flasks in EGM (endothelial cell growth medium) consisting of EBM supplemented with 10 ng/ml epidermal growth factor, 0.4% bovine brain extract, 17.5 microg/ml dibutyryl cyclic AMP, and 1 microg/ml hydrocortisone in the presence of 30% normal human serum. Endothelial cell

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cultures were characterized and determined to be >99% pure on the basis of formation of typical cobblestone monolayers in culture, positive immunostaining for factor VIII-related antigen, and selective uptake of acetylated
5 low density lipoprotein. All experiments were done with HDMEC below passage 10.

Preparation of HDMEC-loaded microcarrier beads (EC-beads)

Gelatin-coated cytodex-3 microcarrier beads were prepared as described by the manufacturer. Approximately
10 80,000 sterile microcarrier beads were washed, resuspended in EGM, and added to approximately 4.5 million HDMEC. The beads and cells were mixed by gentle swirling, incubated at 37°C for 6 hr, and then rotated for 24-36 hr on an orbital mixer in a 37°C oven to
15 generate endothelial cell-loaded microcarrier beads (EC-beads).

Cell migration and capillary sprout formation in fibrin gels and type I collagen gels

A microcarrier in vitro angiogenesis assay
20 previously designed to investigate bovine pulmonary artery endothelial cell angiogenic behavior in bovine fibrin gels (Nehls and Drenckhahn 1995a; Nehls and Drenckhahn 1995b) was modified for the study of human microvascular endothelial cell angiogenesis (Figure 1).
25 Briefly, human fibrogen, isolated as previously described (Mosesson and Finlayson 1963a and 1963b), was dissolved in M199 medium at a concentration of 1.5 mg/ml and sterilized by filtering through a 0.22 μ m filter. Collagen solution was prepared by dissolving sterile type
30 1 collagen in M199 medium at a concentration of 1 mg/ml. In certain experiments, growth factors, such as VEGF, VEGF-C, bFGF or PDGF-BB, were added to the fibrinogen and collagen solutions. In certain experiments, an aliquot of fibrinogen solution was added to collagen solution

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prior to gelation. About 500 EC-beads were then added to the ECM protein solutions, followed by the addition of 0.5 U/ml human thrombin. A 0.3 ml aliquot of each suspension was immediately added to appropriate wells of a 24-well tissue culture plate. After gelation, 1 ml of fresh assay medium (EBM supplemented with 20% normal human serum) was added to each well. The HDMEC angiogenic response was monitored visually and recorded by video image capture. Specifically, capillary sprout formation was observed and recorded with a Nikon Diaphot-TMD inverted microscope (Nikon, Inc., Melville, NY), equipped with a incubator housing with a Nikon NP-2 thermostat and Sheldon #2004 carbon dioxide flow mixer. The microscope was directly interfaced to a video system consisting of a Dage-MTI CCD-72S video camera and Sony 12" PVM-122 video monitor linked to a Macintosh G3 computer. The images were captured at various magnifications using Adobe Photoshop. The effect of angiogenic factors on sprout angiogenesis was quantified visually by determining the number and percent of EC-beads with capillary sprouts. One to two hundred beads (five random low power fields) in each of triplicate wells were counted for each experimental condition. All experiments were repeated at least three times.

25 Porcine Cutaneous Wounds and Immunofluorescence Staining

Porcine cutaneous wounds were harvested at various times and then immunoprobed for expression of integrin receptors as previously described (Clark et al. 1996b). Briefly, full-thickness wounds were made with an 8-mm punch on the backs of White Yorkshire pigs and harvested at the times indicated. Specimens were bisected; one half was fixed in formalin and stained with Masson trichrome, the other half was frozen in liquid nitrogen for immunofluorescence studies. Anti-laminin antibodies

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(Gibco BRL) that were conjugated with biotin were used to identify wound vasculature. All antibodies were used at dilutions that gave maximal specific fluorescence and minimal background fluorescence on frozen tissue

5 specimens. Bound antibody was detected by the avidin-biotin-complex (ABC) technique. Stained specimens were observed and photographed using a Nikon Microphot FXA epifluorescence microscope equipped with a Nikon FX-35DX 35 mm camera.

10 **Confocal microscopy**

Confocal microscopy was done at the University Microscopy Imaging Center, Health Sciences Center, SUNY at Stony Brook, to confirm that sprouts emanating from the EC-beads formed tubes. For these studies EC-beads
15 (150) were suspended in a three dimensional fibrin gel with VEGF (100ng/ml) in a 4-well Lab-Tek Chambered Coverglass and incubated for 7 days. Samples were washed in 2XPBS and then fixed in 2% paraformaldehyde. Images were sequentially obtained (1 micron contiguous
20 tangential cross sections) using a Noran laser scanning confocal system (Odyssey; Noran Instruments, Inc., Middleton, WI) attached to a Nikon Diaphot-TMD microscope. A Silicon Graphics Iris workstation was used for processing digitized micrographs and assembling
25 three-dimensional renditions from confocal images using VoxelView software (Voxelview, Vital Images).

Detailed Reagents.

Solution 1: Endothelial Cell Culture Media (ECCM)

Add to 500 ml EBM: Endothelial Basal Media
30 (Clonetics, San Diego):

75 ml denatured normal human serum (NHS)
(Biowhittaker, Walkersville)

25 ml of 0.35 mg/ml dibutyryl cyclic AMP in EBM
(Sigma, St. Louis)

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2 ml of Bovine Brain Extract (Clonetics)
500 μ l of 1 mg/ml hydrocortisone in ethanol
(Sigma, St. Louis)

500 μ l of 10 μ M mouse EGF (epidermal growth
5 factor) in PBS (Clonetics)

Solution 2: Pretreated Microcarrier Beads

Suspend 1 g (4 million) sterile, gelatin-coated
microcarrier beads (Cytodex-3, Pharmacia, Uppsala,
Sweden) in 50 ml sterile phosphate-buffered saline
10 (PBS, pH 7.4) and incubate at 37°C for 5 hr.

Aspirate the supernatant. Add 50 ml PBS. After
about 10 min of sedimentation, again aspirate the
supernatant, and wash once more with PBS.

Resuspend the beads in 50ml PBS and autoclave
15 at 115°C, 15 psi for 15 min.

Allow the beads to settle. Aspirate the
supernatant, resuspend the beads in 50 ml Solution 1
(ECCM), and store at 4°C for at least 2 days, but
for no longer than one month, prior to use in
20 experiments.

Solution 3: Cell Harvest Solution

Trypsin 0.025% / EDTA 0.01% solution (1 x)
(Clonetics)

Solution 4: Extracellular Matrix Solution (EMS)

25 95% clottable fibrinogen (prepared as described
below) dissolved in EBM (Clonetics) at 1 mg/ml,
sterilized by filtration through 0.2 μ m filter,
aliquotted and stored at -70°C.

Solution 5: Clotting Enzyme Solution

30 0.2 U/ μ l human thrombin (Calbiochem, San Diego), in
sterile water

Solution 6: Angiogenesis Culture Media (ACM)

Add to 200 ml of EBM:

40 ml normal human serum (NHS)

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240 μ l Aprötinin (Sigma, St. Louis) (10 μ g/ μ l dissolved in PBS)

Detailed Culture of human microvascular endothelial cells on microcarrier beads.

- 5 1. Add 15 ml of Solution 1 (ECCM) and 1 ml Solution 2 (beads) to a sterile 50 ml polypropylene conical tube. Stand tube in the hood to sediment the beads. Aspirate the supernatant about 20 min later.
- 10 2. Wash one tissue Culture flask (165 cm²) of 90% confluent human microvascular endothelial cells (4-5 million cells) 3 times with PBS. Add 2 ml of Solution 3 (Trypsin/EDTA). When cells round up/detach, add 8 ml Solution 1 (ECCM) to neutralize Solution 3 (Trypsin/EDTA). Transfer the cell
15 suspension to a 15 ml sterile polypropylene conical tube and centrifuge at 1,000 rpm for 8 min. Aspirate the supernatant and resuspend the cell pellet in 6 ml of Solution 1 (ECCM).
- 20 3. Add the 6 ml of cell solution to the 50 ml tube containing the prepared beads.
4. Gently mix the beads and the cells by swirling the tube. Loosen the cap and incubate the tube in the CO₂ incubator at 37°C. For the initial 6 hr, swirl the tube once every 1.5 hr (4 times).
- 25 5. After incubation for 6 hr, add an additional 25 ml of Solution 1 (ECCM) pre-warmed to 37°C and seal the cap tightly. Load the tube onto the orbital mixer in a 37°C oven, and rotate the tube on the mixer for 24-36 hr. This produces endothelial cell-loaded
30 microcarrier beads (EC-beads).

Note: Before using EC-beads in experiments, quantitate the EC-beads and examine them for quality control. Take a 48-well or 24-well plate, add 500 μ l PBS and add 100 μ l EC-bead solution. Observe whether the bead surfaces are

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covered by relatively confluent microvascular endothelial cells. Count and calculate the concentration of EC-beads.

Detailed Angiogenesis Assay.

1. Thaw an aliquot of Solution 4 at 37°C for 10-15 min.
- 5 2. For each condition to be tested, add 1 ml of Solution 4, with or without the addition of an appropriate concentration of proangiogenic or antiangiogenic factor, to a 1.5 ml sterile Eppendorf tube, and vortex.
- 10 3. Five hundred freshly prepared EC-beads will be needed for each condition to be tested. Add the appropriate number of EC-beads to a 50 ml sterile polypropylene conical tube. Wash twice by gentle sedimentation in 10 ml aliquots of EBM. After
15 aspirating the second wash supernatant, add M μ l of EBM (where $M=50 \times [\text{number of conditions}+2]$) to resuspend the EC-beads.
4. Add 50 μ l of EC-bead preparation to each Eppendorf tube (step 2).
- 20 5. Add 2.5 μ l of Solution 5 (thrombin) to each tube of EC-bead preparation, and immediately mix by pipetting up and down gently three times.
6. For each condition to be tested, add 300 μ l of the appropriate EC-bead preparation to each of
25 triplicate wells in a 24-well tissue culture plate.
7. Allow the plate to remain at room temperature for 20 min to permit proper polymerization of the fibrin gels.
8. Add 1 ml of Solution 6 to each well and incubate the
30 plate at 37°C, 5% CO₂.

In the presence of angiogenic factors, microvascular endothelial cells will invade the fibrin gel and form capillary sprouts within 48-72 hrs, and form capillary networks within 5 days. Negative control (no added

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factor) and positive control (VEGF, 100 ng/ml) should be included in every experiment.

EXAMPLE I

5 Preparation of Fibrinogen

The following discussion of the preparation of fibrinogen is taken from co-pending, co-assigned U.S. Patent Application Serial No. 09/500,512, filed February 9, 2000, the contents of which are hereby incorporated by
10 reference.

The plasma employed in the preparation of fibrinogen is collected by conventional methods and, in practice, can be from blood of a single individual, or, alternatively, it can be pooled from multiple
15 individuals.

As indicated below, fibrinogen is isolated from the plasma by precipitation. In particular, the method of precipitation is achieved with glycine and is carried out in a number of steps.

20 As a first step, the plasma is precipitated with glycine to produce a precipitate and a supernatant in a manner known to those of ordinary skill in the art. A preferred method is described in Galanakis (1995). Preferably, precipitation is carried out by adding
25 glycine to the plasma in an amount such that the final concentration of glycine in the plasma/glycine mixture is from about 1.0 to about 2.1 M. Preferably, the glycine is added as dry glycine to the mixture. Once the addition is complete, precipitation is allowed to proceed
30 during incubation. Incubation proceeds at temperatures below room temperature (e.g., refrigeration temperatures), preferably from between about 2 °C and about 7 °C, more preferably about 5 °C. Incubation occurs for from about 30 minutes to about 12 hours,

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preferably about 1 hour, until the precipitate is formed. In practice, it is most convenient to conduct the precipitation by placing the plasma/glycine mixture in a standard refrigerator (i.e., at about 5 °C). After
5 incubation, a precipitate and a supernatant are produced, which can be separated by conventional methods, such as decanting or, preferably, centrifuging at temperatures from between about 2 °C and about 7 °C, preferably about 5 °C. The precipitate will contain
10 about 90% of the fibrinogen from the plasma. The purity of the fibrinogen is above 50%.

If a preparation having high purity content is desired, fibrinogen is further isolated from the precipitate of the first step. A high purity content
15 fibrinogen is defined as fibrinogen having a purity content of about or above 99%.

A second step is used to isolate fibrinogen from the precipitate of the first step. As used herein, second step generally refers to the process of adding buffer to
20 a precipitate to produce a mixture, adding glycine to the mixture to produce a precipitate and a supernatant, and separating the precipitate and supernatant. This second step can be repeated as many times as desired.

Typically, the precipitate produced in the first
25 step is dissolved in a suitable buffer to produce a solution. Preferably, the buffer has a pH of from about 6 to about 8, preferably from about 6.2 to about 7.6, most preferably about 6.4. One suitable buffer for carrying out this process contains about 150 mM of sodium
30 chloride, about 10 mM sodium phosphate, and 100 mM epsilon-aminocaproic acid in water, preferably in sterile water suitable for injection. Other buffers suitable in the present invention include 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The amount of buffer employed to effect

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the dissolution is preferably from about 30% to about 40% of the volume of the original plasma used in the first step. That is, if the precipitate is precipitated in the first step from plasma having a volume of V , the buffer
5 used in this second step preferably has a volume of from about $0.3 V$ to about $0.4 V$ (i.e., between about $3/10$'s of V to about $4/10$'s of V). More preferably, the volume of buffer employed to effect the dissolution of the precipitate is about 35% of the volume of the plasma used
10 in the first step.

Glycine, typically dry (as described above), and at a suitable concentration is then added to the resulting solution in an amount such that the final concentration of glycine in the resulting mixture is from about 1.7 to
15 about 2.2 M and, more preferably, about 2.1 M. The resulting mixture is incubated, preferably at a temperature of from between about 2°C to about 7°C , most preferably at about 5°C , for from about 30 minutes to about 2 hours, preferably for about one hour.

20 As a result, a precipitate and supernatant form, which are separated, preferably by centrifugation at from between about 2°C to about 7°C , most preferably at about 5°C .

This second step is advantageously repeated several
25 times. Preferably, the second step is repeated at least twice. The precipitate at the end of the second step will contain about 60% of the fibrinogen from the plasma. The fibrinogen is at a purity of about 90%.

To produce fibrinogen having a low purity content,
30 fibrinogen can be additionally or alternatively isolated from the supernatant (instead of the precipitate) of the original plasma/glycine mixture of the first step in a manner similar to that described above. As used herein, low purity fibrinogen means fibrinogen having a purity

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content of about or above 95%, but below 99%. The low
purity fibrinogen is produced by adding glycine,
typically dry and at a suitable concentration, to the
supernatant of the first step to produce a mixture. The
5 resulting mixture is incubated, preferably at
refrigerator temperatures, for about 1 hour. As a
result, a precipitate and supernatant form, which are
separated, preferably by centrifugation at refrigerator
temperatures. The precipitate can then be dissolved in
10 an appropriate buffer (e.g., the ones described above),
glycine added (preferably to a final glycine
concentration of about 2.1 M), the mixture incubated, and
the resulting precipitate separated. This process can be
repeated several times.

15 Irrespective of whether the fibrinogen is isolated
as a precipitate from the precipitate or supernatant or
both of the original plasma/glycine mixture described
above, the precipitate can be advantageously further
treated to purify the fibrinogen. Typically, the further
20 treatment includes dissolving the precipitate resulting
from the glycine precipitation(s) described above in an
appropriate buffer (e.g., as described above) to produce
a solution where the precipitate is present in a volume
of 1/2 to 1/3 of the original plasma, with 1/3 being
25 especially preferred, and precipitating this solution.
Preferably, the precipitation is achieved by adding a
compound such as ammonium sulfate to the solution.
Typically, the ammonium sulfate is added as a saturated
solution, and the amount of ammonium sulfate in the
30 solution is about 25 percent of its saturation level.
The resulting solution is redissolved in a suitable
buffer (as described above) and reprecipitated, preferably
with dialysis in 0.3 M NaCl.

Using the method of the present invention, a precipitate fraction of fibrinogen with a purity of greater than 95% (as ascertained by SDS-polyacrylamide gel electrophoresis ("SDS-PAGE")) is obtained. When high
5 purity fibrinogen is desired, using the method of the present invention fibrinogen having a purity of about or above 99% is obtained.

In one embodiment, the preparation of fibrinogen is as follows.

10 *Fibrinogen Preparations from Precipitate*

The fibrinogen preparations of the present invention were prepared from a 1M glycine precipitation formed in the cold (4°C) that had been discarded in a previously described procedure (Galanakis 1995). In particular,
15 glycine was dissolved in pooled human plasma to attain 1 M final concentration and the mixture was allowed to stand on ice for at least one hour. By subsequent centrifugation at 5°C, two fractions were obtained: the precipitate and the supernatant. The precipitate obtained
20 from plasma containing 1 M glycine, 5°C, was redissolved (pH 6.4) and re-precipitated twice with 2.1 M glycine, 5°C. The precipitate was then dissolved, precipitated with $(\text{NH}_4)_2\text{SO}_4$ to 25% saturation, redissolved and exhaustively dialyzed vs. 0.3M NaCl. The final product
25 was stored at -80°C. Greater than 95% purity was ascertained by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels also disclosed the expected doublet of fibrinogen bands I and II indicating that these bands did not differ from those of fibrinogen in unfractionated
30 plasma.

Fibrinogen Preparations from Supernatant

The following preparations were purified from the supernatant produced during precipitation of the fibrinogen precipitate described above.

- 30 -

Sample 1 was isolated as previously described (Galanakis 1995). It was the purest fibrinogen isolate. It had virtually all its alpha chains intact but lacked molecules with gamma chains that have an extended carboxy terminal which constitute approximately 15% of the fibrinogen in plasma. The extended gamma chains are from a splicing mRNA variant. It extends the gamma chain by deleting the last two amino acids of the regular gamma chain and extending it with a 20 amino acid segment.

10 Sample 2 was isolated by a modification (Galanakis 1995) of the procedure of Mosesson and Sherry (1966). Sample 2 was a highly pure fibrinogen isolate, but less pure than Sample 1, in that a minute contaminant of Factor XIII was detectable by biologic activity. It also
15 had virtually all of its alpha chains intact and contained molecules with both extended and non-extended gamma chains.

Sample 3 was the same as Sample 2, but was enriched with soluble fibrin. Fibrin monomer was prepared as
20 described in Galanakis et al. (1987). An amount of fibrin monomer (from a stock solution of 20 to 30 mg/ml, pH 4.5) was added to exceed 10% (mg/mg) of fibrinogen in solution, allowed to equilibrate at 37°C and any clot that formed was removed. The resulting fibrinogen
25 solution was termed fibrin-saturated and used. Care was taken to ascertain that the pH of the fibrinogen solution remained above 6 in storage and during clotting.

Sample 4 was isolated by a modification (Galanakis 1995) of the procedure of Mosesson and Sherry (1966).
30 Sample 4 had a purity similar to that of Sample 3, but contained a major population of molecules (approx. 20 to 30%) which were clottable but had degraded alpha chains.

Sample 5 was isolated by a modification (Galanakis 1995) of the procedure of Mosesson and Sherry (1966).

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Sample 5 was similar to Sample 4 in structure but less pure in that trace, but detectable, amounts of fibronectin and factor XIII were present.

Sample 6 was similar in all respects to Sample 5, with the exception of being enriched with soluble fibrin. Fibrin monomer was prepared and added to Sample 5 as described above for Sample 3.

Sample 7 was isolated from the supernatant produced in Sample 1 above. It contained only minor amounts of fibrinogen, but was rich in plasma lipids.

Empirical observations have shown that a fibroblast migration enhancement (FME) property is present in fibrinogen preparations of relatively low purity and this activity is stable on freeze thawing of such preparations. Conversely, fibrinogen preparations that are of the highest possible purity, such as fibrinogen fraction I-4, DEAEc peak 1 fraction, and others (produced by methods known to those skilled in the art) either possess low or no activity. Of particular use is fraction I-4, which can be prepared in bulk and whose moderate FME activity progressively decreases on repeat freeze thawing, thus enabling its use as a negative control. In the discussion below, positive and negative controls are termed C1 and C2, respectively. Fibrinogen isolates with high activity are isolated from plasma by procedures that yield two kinds of active isolates, one enriched with soluble fibrin and the other lacking fibrin enrichment. The procedures also yield a lipid or lipoprotein rich (L) component which is enriched in FME activity.

Fibrin-rich fibrinogen preparation.

Glycine is dissolved in plasma to 1 M (or 1 molar) concentration and allowed to stand at 4° C overnight. The precipitate formed is dissolved in phosphate buffered

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saline, pH 6.4, subjected to reprecipitation with 2.1 M Glycine at 4° C, and this step is repeated. An additional precipitation step is performed with either 2.1 M Gly or 25% saturated Ammonium Sulfate. The fibrinogen isolate thus obtained is dialyzed vs 0.3 M NaCl at 4° C and termed 2F. As shown below, fibrinogen 2F may be further separated into sub-fractions. A large amount of insoluble fibrin gel forms during dialysis, and this is removed by centrifugation as described below.

10 *Fibrinogen low in fibrin content preparation.*

An isolate is obtained from the plasma supernatant of the initial 1 M Gly step detailed in the paragraph above. For this purpose, additional Glycine is added and dissolved to achieve 2.1 M Gly concentration and the precipitate obtained at 4° C is subjected to the same precipitation steps described above. The final isolate is dialyzed as above and termed 2H. As shown below, fibrinogen 2H may be further separated into sub-fractions. During dialysis a small amount of insoluble fibrin forms and is removed by centrifugation as described in the paragraph below.

Further sub-fractionation to obtain and characterize sub-fractions.

Each of the fractions, 2F and 2H, is subjected to centrifugation using at least 4000 XG for 30 or more minutes at 4° C. The resulting three sub-fractions are illustrated in Fig. 1. One sub-fraction is a lipid or lipoprotein component which contains insoluble material floating at the top of the solution. This subfraction is termed 2FL (from the 2F fibrin rich fraction) or 2HL (from the 2H low fibrin content fraction). Use of a spatula or other implement permits harvesting these sub-fractions. The second sub-fraction is the bulk of isolated fibrinogen and is referred to herein as 2F sub-

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fraction (fibrin rich fibrinogen) or 2H sub-fraction (low fibrin content fibrinogen). The third sub-fraction is a pellet at the bottom of the centrifuged solution, which consists of insoluble fibrin gel and is discarded.

- 5 During the harvest and testing of the lipid and fibrinogen sub-fractions certain characteristics emerge. One is that fibrinogen 2H invariably contains major amounts of sub-fraction 2HL, and fibrinogen 2F, by contrast, tends to contain lower amounts of sub-fraction
- 10 2FL. Such differences are demonstrable by dissolving the material and measuring its turbidity. Further, some 2FL sub-fractions are of substantial amounts while others are of very small amounts compared to those in the 2HL counterparts from the same starting plasma. A
- 15 constellation of characteristics of the L isolates is their self-evident low density and coalescence into insoluble floating sheets or particles following centrifugation, their yellowish-white and opaque appearance on visual inspection (particularly marked in
- 20 2HL), their capacity to be readily dispersed and re-dissolved in fibrinogen containing buffers, and their high turbidity when re-dissolved and assessed spectrophotometrically.

To compare the FME activity of 2HL or 2FL with their

25 2H or 2F counterparts, fibrin is formed in the assay from the same lot. Comparison can also be made with the same parent (or uncentrifuged fibrinogen preparation). In order to test the L moiety of 2FL or 2HL alone, fibrinogen can be removed so that the sub-fraction can be

30 tested without its parent fibrinogen. To remove its fibrinogen, ammonium sulfate is added to 25% saturation and the precipitate is discarded. The floating insoluble material on the top of the solution is then harvested and dialyzed.

An alternate method to sub-fractionate 2F or 2H isolates.

The fractions can be sub-fractionated by subjecting them to size exclusion chromatography, as shown in Fig.

2. This enables removal of most of the lipid and of the
5 soluble fibrin components, so that each such component
can be tested. Absorbance values of the eluting
fractions are obtained at 280 nm and at 350 nm.

Absorbance at 280 nm reflects the presence of protein and
that at 350 nm reflects light scattering and, thus, the
10 opacity caused by the lipid content of the fibrinogen
solution. This chromatographic procedure results in an
early elution peak, labeled peak I. Fractions containing
this peak appear white-opaque, show high absorbance
values at 350 nm as expected, and show the presence of
15 protein by their absorbance values at 280 nm. When
dialyzed against water and freeze dried, this peak is
insoluble in buffer but can be resolubilized at least in
part in fibrinogen or other protein solutions. The
second peak, labeled peak II, consists of fibrinogen and
20 soluble fibrin. This peak shows absorbance at 280 nm but
little or negligible absorbance at 350 nm, as shown, and
constitutes the bulk of protein applied to the column.

*Fibroblast enhancing (FME) activity: 2F, 2H and non-
chromatographic subfractions.*

25 Fractions 2F and 2H have ample activity, with 2H
showing moderately higher activity than 2F. Similarly,
this activity remains high, as shown in Fig. 3, in both
the L rich subfractions (2FL, 2HL) and fibrin rich sub-
fractions (2H, 2F). Moreover, these sub-fractions, 2FL
30 and 2HL, show similarly high activity (See Fig. 3 insert)
and this activity remains when fibrinogen is removed from
2HL and 2FL (not shown). Because insoluble lipid in 2HL
may induce formation of an abnormal fibrin matrix, care
is taken to avoid insoluble lipid aggregates when using

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2HL. This is not the case with 2FL, which contains a much lower lipid content and lacks such large insoluble aggregates.

Fibroblast enhancing (FME) activity: Chromatographic
5 *subfractions.*

Tested as outlined above, both peaks (shown in Fig. 2) possess substantial fibroblast migration enhancing activity. The fibroblast migration activity of the lipoprotein (L) peak of Fig. 2 is shown in the Fig. 4.

10 This is consistent with the results from the post-centrifugation sub-fractions described above, indicating that the activity is present both in the L rich and the fibrinogen (L poor) peaks. Considering the small amount of L sub-fraction required to demonstrate the activity,

15 this implies the lipid in these isolates contains relatively higher activity than does fibrinogen per se. That is to say, the activity is more lipophilic than fibrinogenophilic. Moreover, when fibrin-rich fibrinogen was isolated from the ascending limb of the chromatogram

20 it too displayed substantial activity, not shown, consistent with the fact that fraction 2F is fibrin rich and shows activity comparable to 2H.

Summary and conclusions.

The fibroblast migration enhancement activity of

25 fibrinogen isolated by the above procedures is associated with three components or sub-fractions: fibrin rich fibrinogen, lipid rich fibrinogen, and fibrinogen not enriched with either fibrin or lipid. Although lipid rich fibrinogen (2FL or 2HL) displays somewhat higher

30 activity, this activity remained when the lipid component was rendered free of fibrinogen by chromatography or other means. Moreover, fibrin rich fibrinogen which is also rendered lipid poor clearly retains its high activity. In another set of observations (data not

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shown), this activity is progressively lost by freeze thawing of I-4, and absent in DEAEc pure fibrinogen. Taken together, these results imply a non-fibrinogen and possibly non-lipid hydrophobic agent whose FME activity
5 remains stable in storage of fibrinogen/lipid mixtures. This explains its stability in fibrinogen isolates of relatively low purity (i.e. $\geq 95\%$ by protein measurements), and enables potential use of such preparations in situations where enhancement of wound
10 healing is clinically important. Also, in order for fibrinogen isolates to possess the highest FME activity they need be enriched with substantial amounts of a lipid component that co-isolates with them from normal plasma and results also in stability of this activity when
15 fibrinogen is stored frozen and re-frozen. What is more, the lipid component can be isolated and re-introduced into fibrinogen of high purity or any other fibrinogen isolate for the purpose of further enriching its FME activity. This discovery makes it possible to monitor
20 the amounts of this lipid component in any fibrinogen and/or soluble fibrin preparations.

EXAMPLE II

Characterization of in vitro angiogenesis of HDMEC in 25 three-dimensional fibrin

To study the regulation of human angiogenesis, a relatively simple, reproducible, three-dimensional in vitro angiogenesis assay for HDMEC was established by modification of a previously described in vitro bovine
30 angiogenesis model (Nehls and Drenckhahn 1995a; Nehls and Drenckhahn 1995b) (Figure 1). In this assay, HDMEC cultured on the surface of microcarrier beads (EC-beads) are embedded in three-dimensional fibrin, collagen, or fibrin/collagen gels with or without the addition of an

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angiogenesis factor. When an angiogenesis factor was added to fibrin gel containing EC-beads, HDMEC formed capillary sprout-like structures, which projected from the surface of the EC-beads and invaded into the fibrin gel within 48 hr (Figure 2b). Note that the base of each sprout was in intimate contact with the surface of the EC-bead (Figure 2b). By 7 days the endothelial sprouts had elongated and in some cases formed branching capillary tube-like structures (Figure 2c). In contrast, without the addition of an angiogenesis factor, no significant HDMEC sprout formation occurred from the surface of EC-beads in fibrin gel, despite the presence of 20% normal human serum in the medium (Figure 2a).

To demonstrate whether capillary tube-like structures (Figure 3A) had lumina, reflective confocal microscopic analysis was used. Computer-assisted sectioning clearly revealed the presence of a lumen (Figure 3B) in the capillary-like structure shown in Figure 3A. As the tube was cut through by a series of 1 micron contiguous tangential cross sections, first the top and then the central lumen with two walls become visible (Figure 3B). Thus, critical steps of human angiogenesis, i.e. capillary sprout formation, migration and invasion of a 3-dimensional human ECM, and capillary tube formation, occurred in the in vitro system using human microvascular endothelial cells.

Because, in the absence of added angiogenesis factor, no HDMEC sprouts emanated from the EC-bead surface into the surrounding fibrin gel (Figure 2a), the degree of HDMEC angiogenesis response to increasing concentrations of angiogenesis factor could be easily quantified by determination of the number of beads with sprout formation. In addition the length of individual sprouts/tubes, and the degree of network formation can be

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easily quantified. VEGF (0 ng/ml - 100 ng/ml) induced a positive dose-response of sprout angiogenesis at 48 hr (Figure 4). Moreover, in the presence of 100 ng/ml VEGF, a capillary-like network formed by 7 days (Figure 5a).

- 5 When observed at high magnification, these capillary-like structures appeared to be composed of linear arrays of multiple HDMEC (Figure 5b). Networks (Figure 5c) formed by branching and fusion of capillary tubes from adjacent beads (Figure 5d).

10

EXAMPLE III

Induction of HDMEC capillary sprout formation by VEGF, VEGF-C, bFGF and PDGF-BB

- Considerable evidence demonstrates that VEGF, VEGF-
15 C, bFGF and PDGF-BB are involved in stimulating angiogenesis in vivo (Oh et al. 1997; Friedlander et al. 1995; Risau et al. 1992). However, their direct angiogenic effect on human microvascular endothelial cells has not yet been well defined in vitro. The effect
20 of each of these factors on capillary sprout formation by HDMEC in fibrin gel was determined at 48 hr and quantified by calculating the percent of beads with sprouts. VEGF-C, a member of the VEGF family, recently has been determined to be an important growth factor for
25 the lymphatic vascular system that has potent angiogenic effects in vivo (Oh et al. 1997). Similar to VEGF, VEGF-C dose-dependently induced sprout angiogenesis of HDMEC in fibrin (Figure 6). However, its stimulatory effect was markedly less than that of VEGF, with maximal
30 response at 100 ng/ml only about 50% of the response to VEGF. bFGF strongly induced sprout angiogenesis of HDMEC in fibrin (Figure 7), with maximal response at 30 ng/ml comparable to that of VEGF at 100 ng/ml. The angiogenic stimulatory effect declined at higher concentrations of

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bFGF (50-100 ng/ml). Although PDGF-BB has been reported to be involved in angiogenesis in vivo (Risau et al. 1992), it did not significantly induce sprout angiogenesis of HDMEC in fibrin at concentrations ranging from 10 to 100 ng/ml (Figure 7). Thus, VEGF, VEGF-C, bFGF and PDGF-BB differentially regulated sprout angiogenesis of HDMEC in fibrin in vitro. VEGF and bFGF were strong direct angiogenic factors for HDMEC in fibrin.

10

EXAMPLE IV

Differential regulation of angiogenic response of HDMEC by fibrin and collagen gel

The process of angiogenesis is a dynamic interaction of microvascular endothelial cells with angiogenesis factors and the surrounding ECM milieu (Madri et al. 1996). To understand the relationship between angiogenesis and different ECM components during granulation tissue formation of wound repair, tissue specimens from 5, 7 and 10 day porcine wounds were analyzed. Previously other investigators have shown that 5 day wounds are mainly composed of a fibrin-rich provisional matrix, whereas 7 day wounds have a substantial organized collagen fiber network, and 10 days wounds have developed a compacted contracted collagen scar (Clark et al. 1982a and 1982b; Welch et al. 1990; Clark et al. 1995). Staining of 5, 7, and 10 day wound specimens with Masson trichrome (Figure 8a, c, e) and with anti-laminin, as a marker for blood vessels (Figure 8b,d,f), revealed that the fibrin-rich early granulation tissue in 5 day wounds is filled with newly formed vessels (Figure 8a,b). These neovessels consistently stained weakly for laminin, most likely as a result of blood vessel immaturity. Such weak staining for laminin

in immature blood vessels was previously observed in the microvasculature of human fetal skin (Tonnesen et al. 1985). By 7 days, the maturing blood vessels form an organized vertical array as collagen accumulates in the wound ECM (Figure 8c,d). At 10 days, as the collagen bundles thicken to produce scar, many blood vessels are regressing (Figure 8e,f). Thus, during wound repair in vivo, the angiogenic neovessels in early granulation tissue mature and then regress as fibrin is replaced by collagen in the wound space (Figure 9).

To determine whether fibrin and collagen differentially regulated the angiogenic response of microvascular endothelial cells to angiogenic factors, the angiogenic response of HDMEC in 3-dimensional fibrin and collagen gels in response to VEGF was compared, since VEGF has been reported to be abundantly present in early wounds, but not in normal skin (Brown et al. 1992). EC-beads were embedded in fibrin gel or in collagen gel, with or without the addition of VEGF for 48 hr. In the absence of VEGF, HDMEC remained on the surface of EC-beads and did not invade either fibrin gel (Figure 10a) or collagen gel (Figure 10b). In the presence of VEGF in fibrin gel, HDMEC formed capillary-like sprouts from the surface of EC-beads and invaded and migrated into the surrounding fibrin (Figure 10c). In contrast, when VEGF was added to collagen gel, HDMEC invaded and migrated into the surrounding collagen as individual cells, but did not form sprouts (Figure 10d).

To confirm that in this 3-dimensional ECM system, invasive angiogenic sprout formation induced by VEGF depended on the presence of fibrin, EC-beads were embedded in collagen admixed with fibrin and the angiogenic response to that in either pure fibrin or pure collagen gel was compared. In collagen gel without

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fibrin (Figure 11a), VEGF induced individual cell invasion and migration, as previously observed. With addition of fibrin to collagen gel (Figure 11b, 17% fibrin, and Figure 11c, 29% fibrin), sprout formation
5 occurred together with individual cell invasion and migration. In pure fibrin (Figure 11d), VEGF induced sprout angiogenesis. Thus the presence of fibrin appeared to be essential for HDMEC sprout angiogenesis induced by VEGF.

10

EXAMPLE V

Promotion of Angiogenesis and Vasculogenesis Using Cultured Endothelial Cells

Cultured endothelial cells grow well as aggregates.
15 When in the proper environment, these aggregates can form capillary-like tubes. This in vitro phenomenon simulates vasculogenesis as observed during fetal development. Vasculogenesis in tissues can be promoted by implanting endothelial cell aggregates, and angiogenesis in tissues
20 can be promoted by implanting endothelial cells on microcarriers.

An endothelial cell microcarrier system using dextran beads is described in the Materials and Methods, and Examples II-IV. Capillary-like tubes sprout from
25 these beads when they are placed in the proper environment. No capillary sprouts are observed when beads loaded with endothelial cells are placed in either fibrin gels or type 1 collagen gels without an angiogenesis stimulator. When vascular endothelial cell
30 growth factor (VEGF) is present in the fibrin gel, capillary tubes sprout from the beads. In contrast, no sprouts are observed when VEGF is added to collagen gels. Instead, single endothelial cells migrate off of the beads into the surrounding 3-dimensional collagen matrix.

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In accordance with the subject invention, endothelial cell aggregates or endothelial cells on microcarriers are added to wounds or other tissues that need capillary bed enhancement. The endothelial cell aggregates or endothelial cells on microcarriers can also be added to artificial tissue constructs to promote vasculogenesis or angiogenesis, respectively, within these constructs. Examples of artificial skin constructs includes systems produced by Organogenesis (Boston, MA), Ortec (NY, NY), and Integra Life Sciences (Princeton, NJ).

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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What Is Claimed Is:

- 1 1. A cellular matrix system comprising:
2 cells of a selected cell type disposed on
3 microcarrier particles; and
4 an in vitro defined matrix having the microcarrier
5 particles disposed therein, wherein the in vitro matrix
6 simulates a natural environment in which the cells
7 naturally reside.
- 1 2. The cellular matrix system of claim 1 wherein
2 the cells of a selected cell type are endothelial cells.
- 1 3. The cellular matrix system of claim 2 wherein
2 the endothelial cells are cultured human dermal
3 microvascular endothelial cells.
- 1 4. The cellular matrix system of claim 2 wherein
2 the in vitro defined matrix is a fibrin matrix.
- 1 5. The cellular matrix system of claim 2 wherein
2 the in vitro defined matrix is a collagen matrix.
- 1 6. The cellular matrix system of claim 1 wherein
2 the microcarrier particles are pre-conditioned.
- 1 7. The cellular matrix system of claim 1 wherein
2 the microcarrier particles are bioresorbable.
- 1 8. The cellular matrix system of claim 1 further
2 comprising cells of a different selected cell type
3 overlaid by or admixed with the in vitro defined matrix
4 having the microcarrier particles disposed therein.

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1 9. The cellular matrix system of claim 8 wherein
2 the cells of a different selected cell type are tumor
3 cells.

1 10. The cellular matrix system of claim 8 wherein
2 the cells of a different selected cell type are provided
3 as a layer of cells, and wherein the in vitro defined
4 matrix overlies the layer.

1 11. The cellular matrix system of claim 8 wherein
2 the cells of a different selected cell type are provided
3 as a droplet or aggregate of cells, and wherein the in
4 vitro defined matrix overlies or is admixed with the
5 droplet or aggregate.

1 12. A cellular angiogenesis system comprising:
2 endothelial cells disposed on microcarrier
3 particles; and
4 an in vitro defined fibrin matrix having the
5 microcarrier particles disposed therein.

1 13. A method of determining the effect on cellular
2 migration/angiogenesis of a compound or effector cell of
3 interest, the method comprising:
4 providing cells of a selected cell type on
5 microcarrier particles which are disposed in an in vitro
6 defined matrix, wherein the in vitro defined matrix
7 simulates a natural environment in which the cells
8 naturally reside;
9 determining migration/angiogenesis of the cells into
10 the in vitro defined matrix;
11 including a compound or effector cell of interest in
12 the in vitro defined matrix;

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13 redetermining migration/angiogenesis of the cells
14 into the in vitro defined matrix which includes the
15 compound or effector cell of interest; and
16 determining the effect on cellular
17 migration/angiogenesis of the compound or effector cell
18 of interest.

1 14. The method of claim 13 wherein the cells of a
2 selected cell type are endothelial cells.

1 15. The method of claim 14 wherein the endothelial
2 cells are cultured human dermal microvascular endothelial
3 cells.

1 16. The method of claim 14 wherein the in vitro
2 matrix is a fibrin matrix.

1 17. The method of claim 14 wherein the in vitro
2 defined matrix is a collagen matrix.

1 18. The method of claim 13 wherein the microcarrier
2 particles are pre-conditioned.

1 19. The method of claim 13 wherein the microcarrier
2 particles are bioresorbable.

1 20. The method of claim 13 further comprising
2 overlaying or admixing with the in vitro defined matrix
3 having the microcarrier particles disposed therein cells
4 of a different selected cell type, and detecting
5 migration/angiogenesis of the cells of the selected cell
6 type into the cells of the different selected cell type.

1 21. The method of claim 20 wherein the cells of the
2 different selected cell type are tumor cells.

1 22. The method of claim 20 wherein the cells of the
2 different selected cell type are provided as a layer of
3 cells, and wherein the in vitro defined matrix overlies
4 the layer.

1 23. The method of claim 20 wherein the cells of the
2 different selected cell type are provided as a droplet or
3 aggregate, and wherein the in vitro defined matrix
4 overlies or is admixed with the droplet or aggregate.

1 24. The method of claim 13 wherein the compound or
2 effector cell of interest stimulates
3 migration/angiogenesis.

1 25. A method of determining the effect on
2 angiogenesis of a compound or effector cell of interest,
3 the method comprising:
4 providing endothelial cells on microcarrier
5 particles which are disposed in an in vitro defined
6 fibrin matrix;
7 determining angiogenesis of the cells into the in
8 vitro defined fibrin matrix;
9 including a compound or effector cell of interest in
10 the in vitro defined fibrin matrix;
11 redetermining angiogenesis of the cells into the in
12 vitro defined fibrin matrix which includes the compound
13 or effector cell of interest; and
14 determining the effect on angiogenesis of the
15 compound or effector cell of interest.

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1 26. A method of promoting angiogenesis in a tissue,
2 the method comprising:
3 selecting a tissue in which angiogenesis is desired;
4 and
5 introducing cultured endothelial cells into the
6 tissue to promote angiogenesis in the tissue.

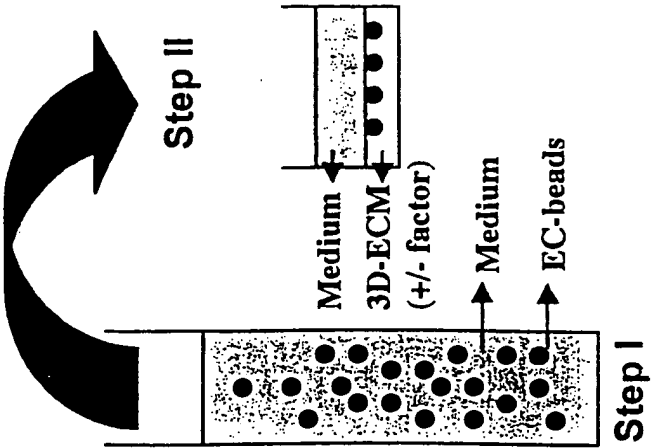
1 27. The method of claim 26 wherein the tissue is a
2 wound.

1 28. The method of claim 26 wherein the tissue is an
2 artificial tissue.

1 29. The method of claim 26 wherein the cultured
2 endothelial cells are introduced as an endothelial cell
3 aggregate.

1 30. The method of claim 26 wherein the cultured
2 endothelial cells are introduced as endothelial cells
3 disposed on microcarrier particles.

Fig. 1



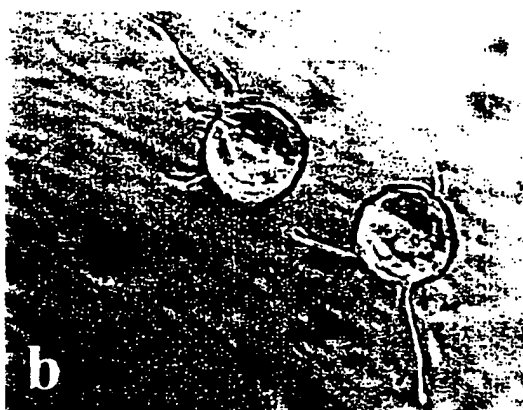
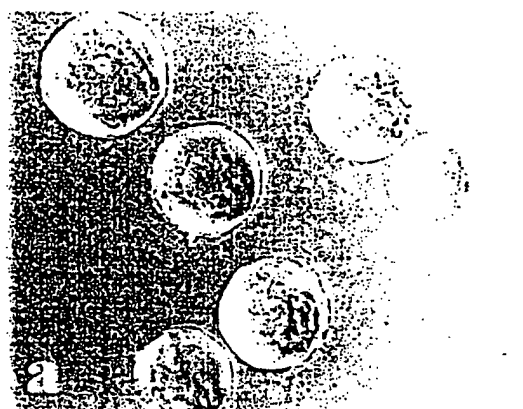
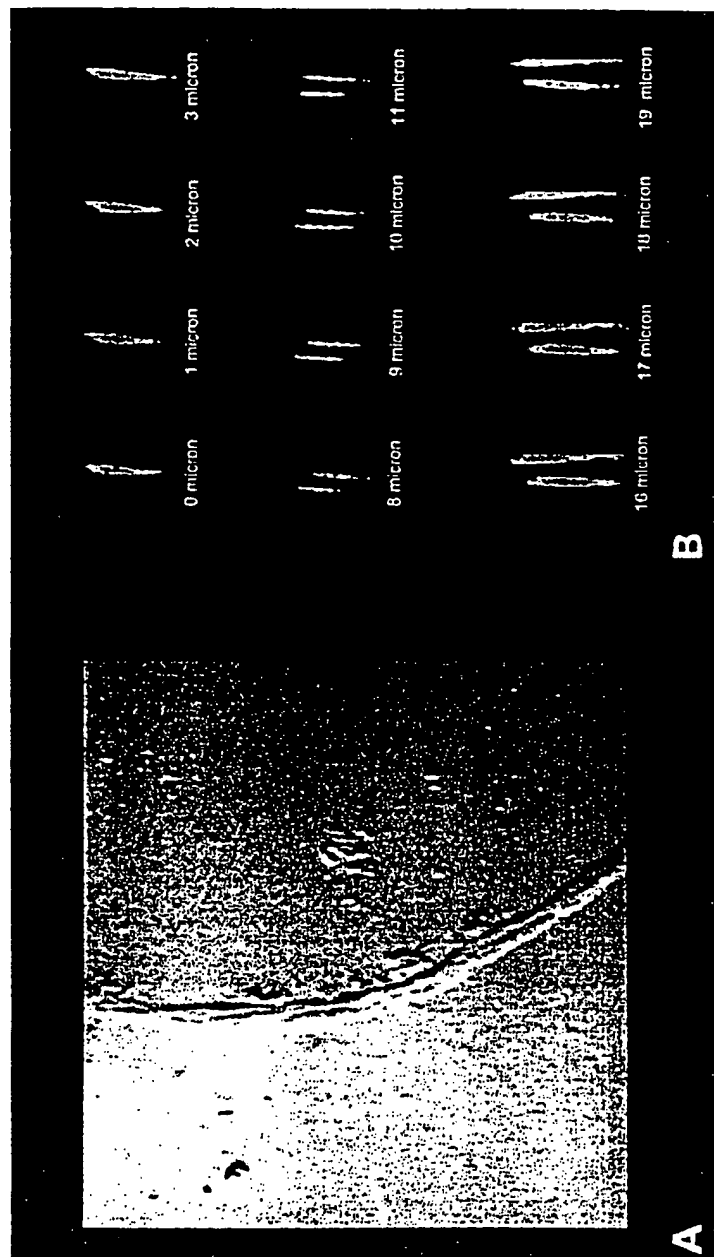


Fig. 2

Fig. 3



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Fig. 4

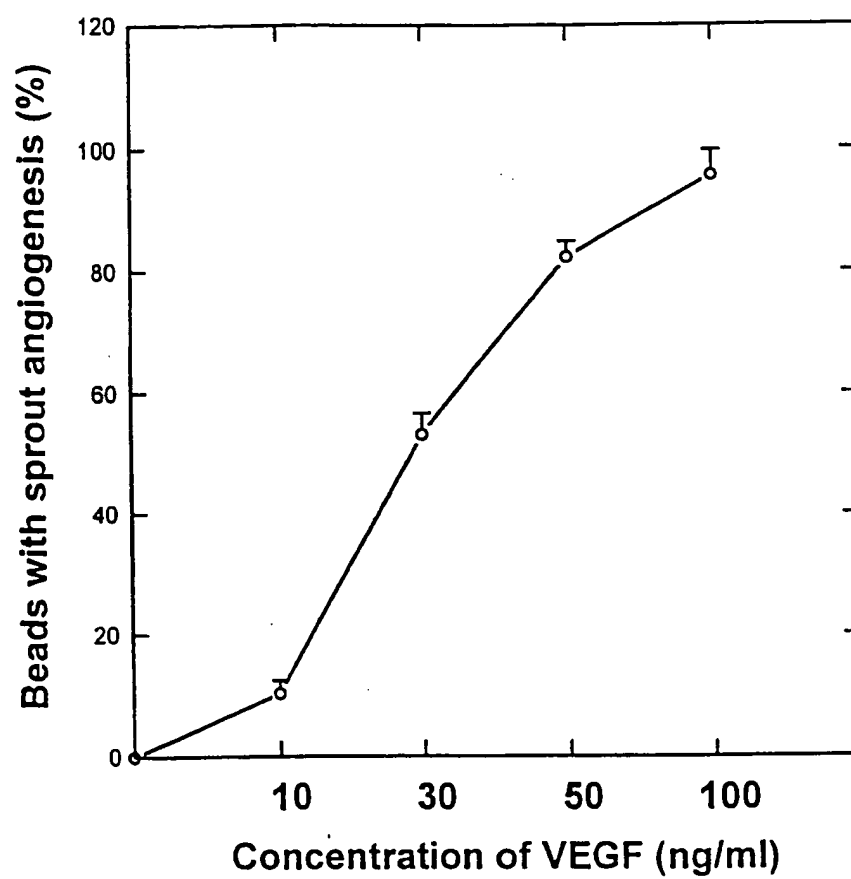
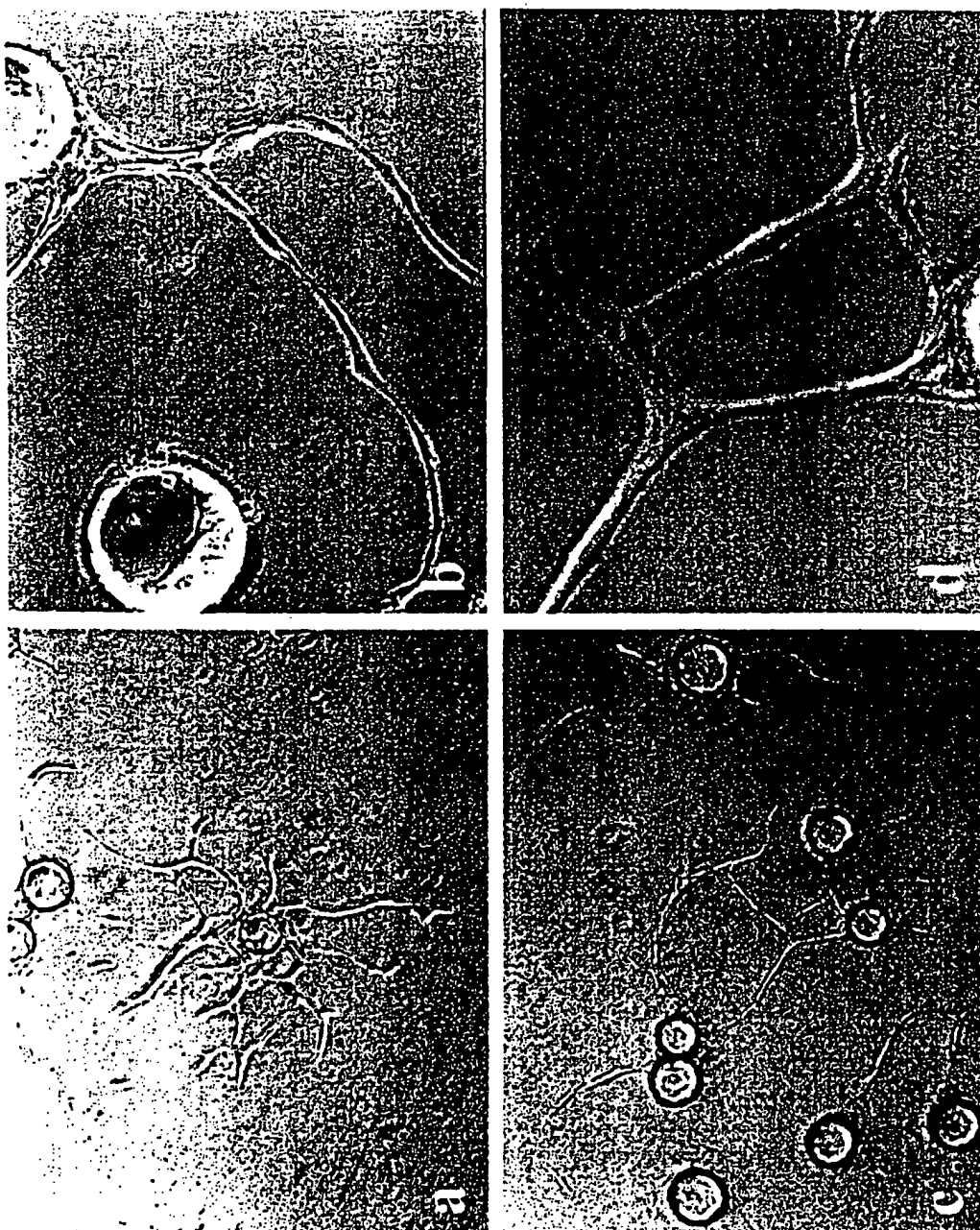


Fig. 5



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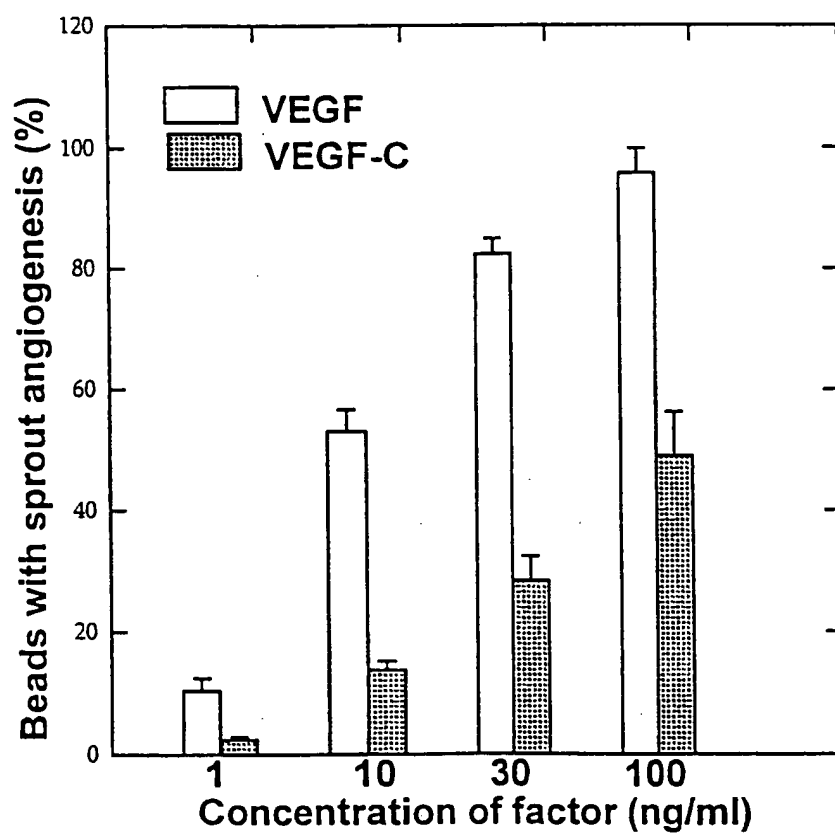


Fig. 6

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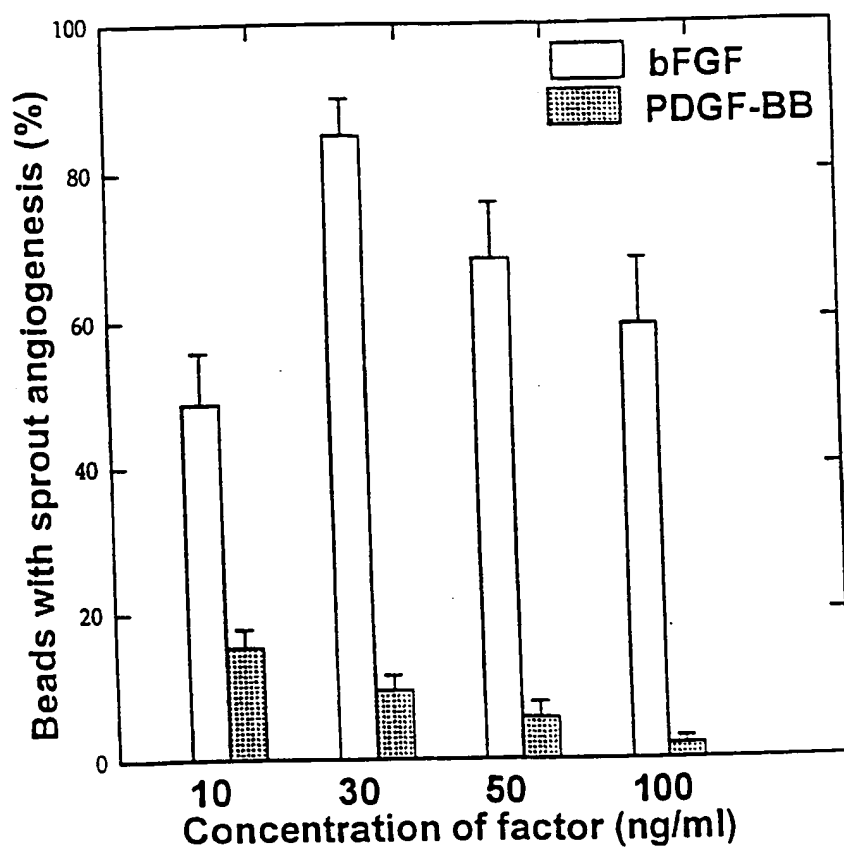


Fig. 7

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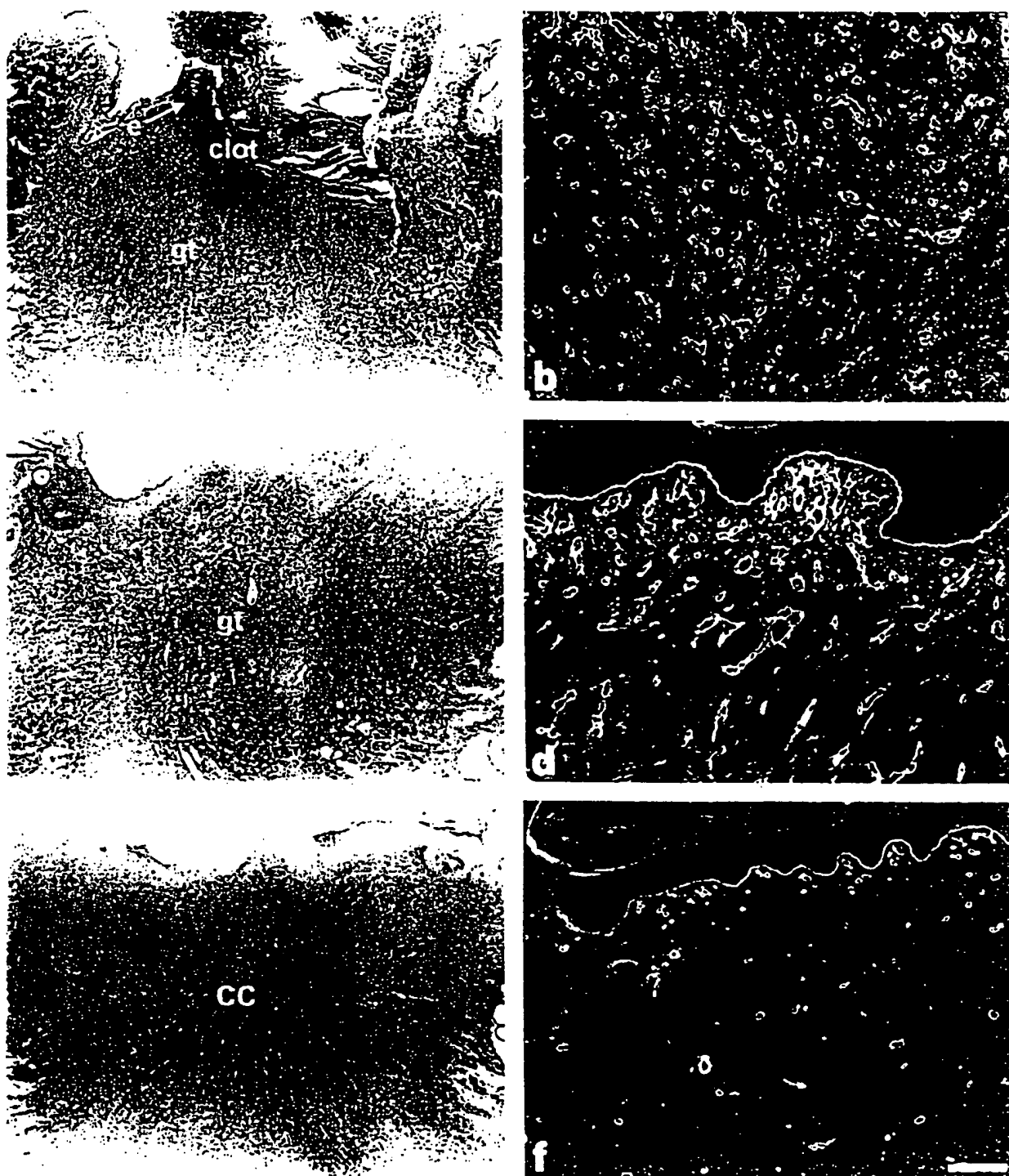


Fig. 8

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Fig. 9

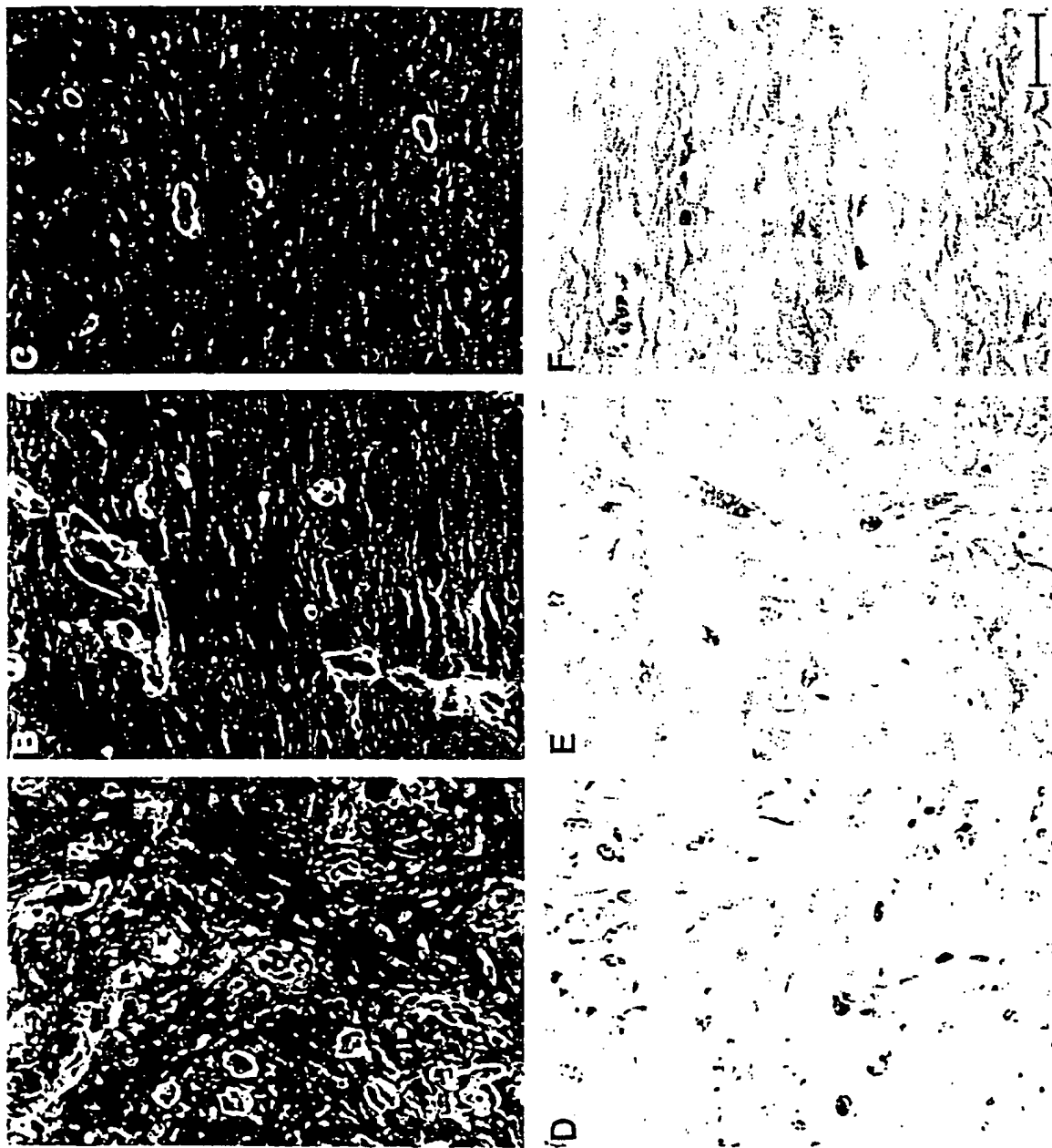


Fig. 10

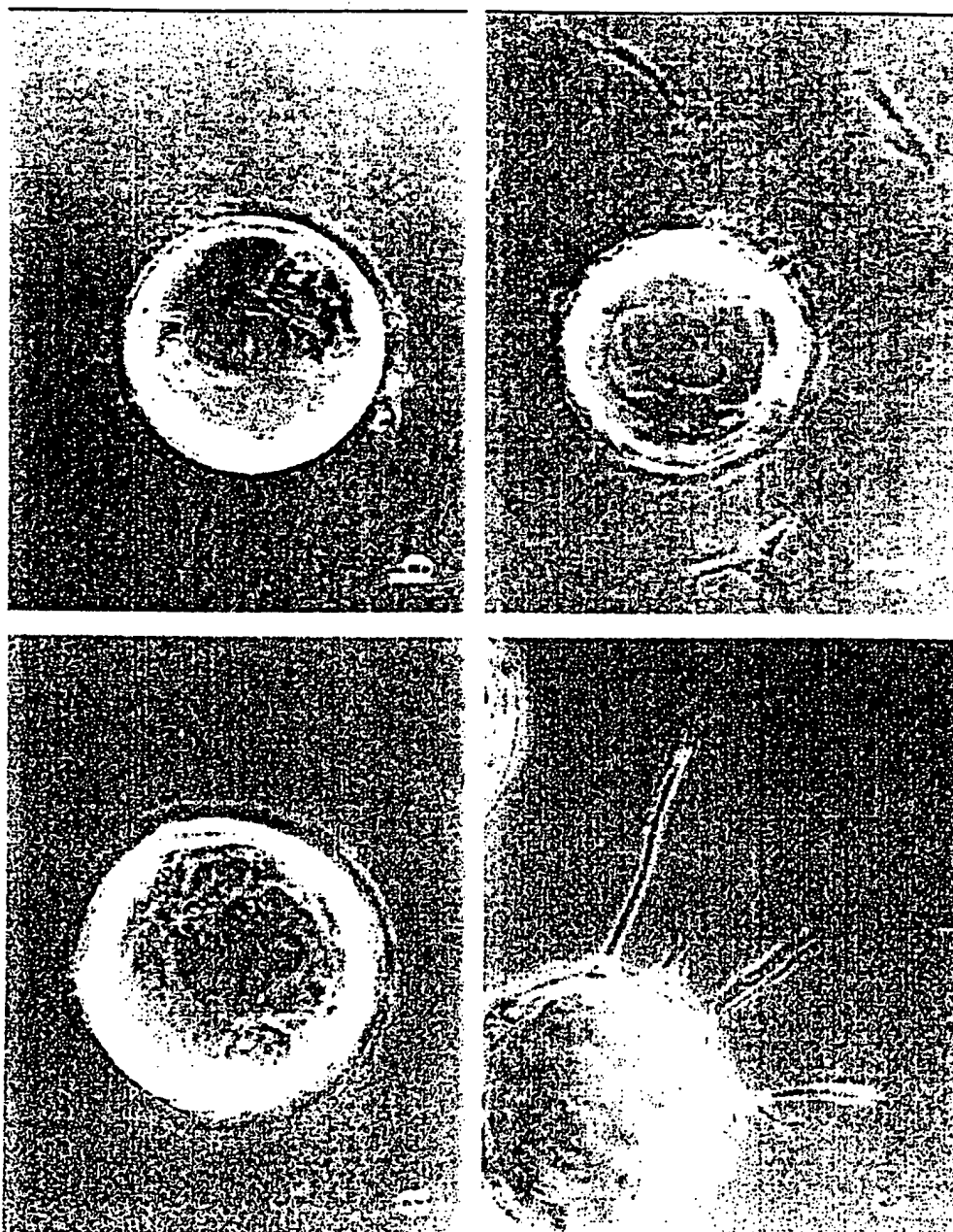
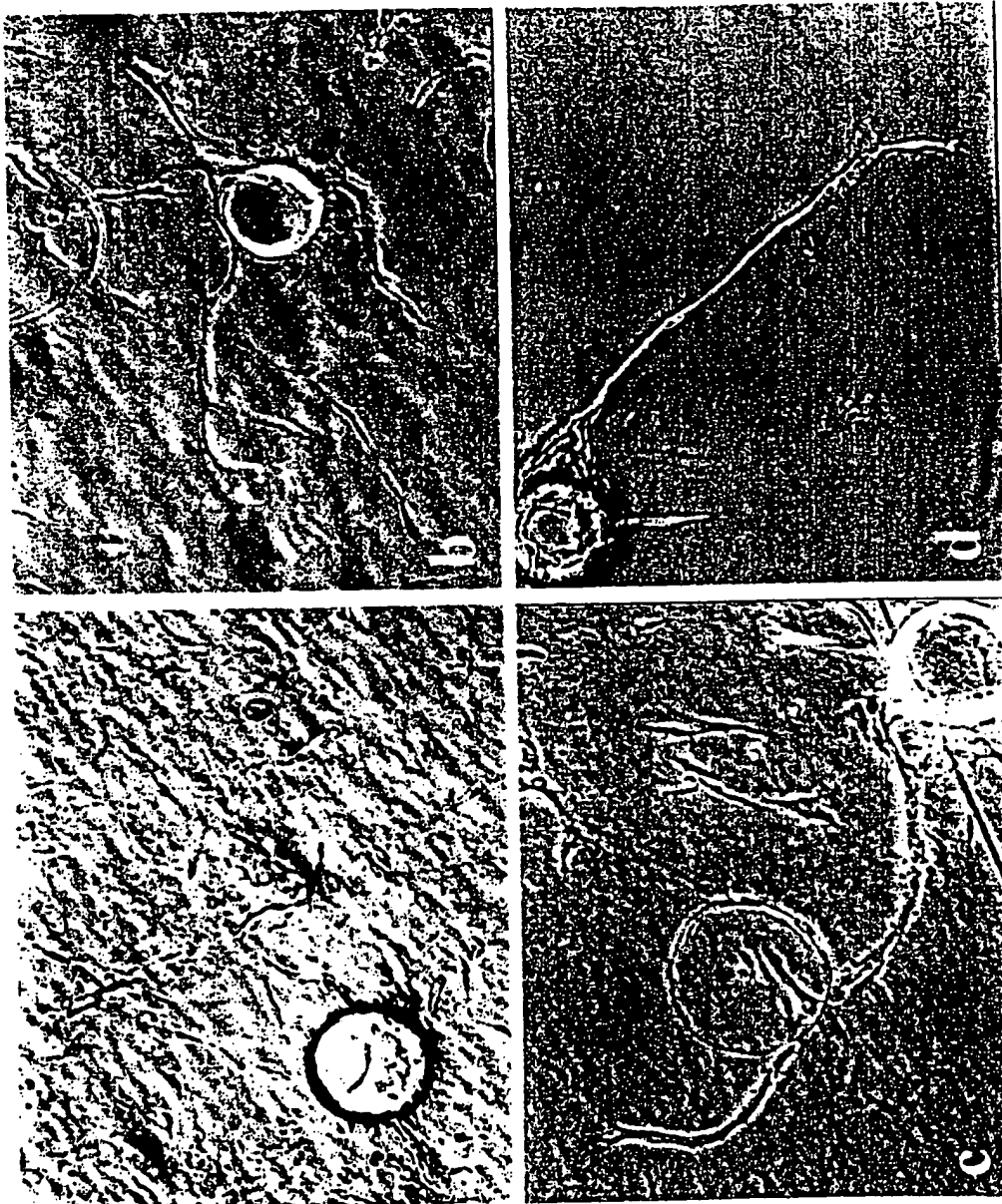


Fig. 11



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/10091

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61L27/22 A61L27/24 A61L27/38 A61L27/60 C12N11/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 10217 A (VITAPHORE WOUND HEALING INC) 25 June 1992 (1992-06-25) abstract; claims	1-3,5-7, 13-15, 17-20, 22,24, 26,27,30
X	US 5 804 178 A (JOHNSON LYNT ET AL) 8 September 1998 (1998-09-08) abstract column 5, line 61; claims -/-	1,2,5-8, 10,13, 14, 17-20, 22,24, 26-28,30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

14 July 2000

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EP 0 518 389 A (UNIV JEFFERSON) 16 December 1992 (1992-12-16)</p> <p>abstract page 14, line 8 - line 26; claims -----</p>	<p>1-7, 12-20, 22, 24-26, 28,30</p>

INTERNATIONAL SEARCH REPORT

Internal application No.

SAE 280257

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 26-30 (please see remark)
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 26-30 are directed to a therapeutic method of treatment of (diagnostic method practised on) the human/animal body, the search report has been carried out and based on the alleged effects of the compound/

2. ☐ Claims Nos.: composition (see PCT-Rule 39.1 (iv)).
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/10091

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(51) International Patent Classification⁷: A61L 27/22,
27/24, 27/38, 27/60, C12N 11/00

(21) International Application Number: PCT/US00/10091

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: CELLULAR MATRIX SYSTEM AND USE THEREOF

(57) Abstract: The invention provides a cellular matrix system which comprises cells disposed on microcarrier particles, and an in vitro defined matrix having the microcarrier particles disposed therein. The invention further provides a method for determining the effect on cellular migration/angiogenesis of a compound or effector cell of interest. The method comprises providing cells on microcarrier particles which are disposed in an in vitro defined matrix which simulates a natural environment in which the cells naturally reside; determining migration/angiogenesis of the cells into the in vitro defined matrix; including a compound or effector cell of interest in the in vitro defined matrix; redetermining migration/angiogenesis of the cells into the in vitro defined matrix which includes the compound or effector cell of interest; and determining the effect on cellular migration/angiogenesis of the compound or effector cell of interest. The invention also provides a method of promoting angiogenesis in a tissue. The method comprises selecting a tissue in which angiogenesis is desired; and introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue.

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**CELLULAR MATRIX SYSTEM
AND USE THEREOF**

This application claims priority of U.S. Provisional
5 Patent Application No. 60/129,507, filed April 15, 1999.

This invention was made with support under NIA Grant
No. AG 101143-12. The U.S. Government may have certain
rights in this invention.

10

FIELD OF THE INVENTION

The subject invention is directed generally to
cellular migration/angiogenesis, and more particularly to
a cellular matrix system comprising cells disposed on
15 microcarrier particles which are disposed within an in
vitro defined matrix, and to a method using this system
to determine the effect on cellular
migration/angiogenesis of a compound or effector cell of
interest.

20

BACKGROUND OF THE INVENTION

Throughout this application various publications are
referenced, many in parenthesis. Full citations for each
of these publications are provided at the end of the
25 Detailed Description. The disclosures of each of these
publications in their entireties are hereby incorporated
by reference in this application.

Angiogenesis, the generation of new capillaries from
pre-existing blood vessels, is critical for various
30 fundamental physiological and pathological processes such
as wound healing, tumor growth and morphogenesis (Folkman
and Shing 1992). During normal physiologic events of
morphogenesis and tissue repair, angiogenesis is tightly
regulated temporally and spatially. For example, in the
35 initial phase of cutaneous wound repair, endothelial
cells invade the fibrin/fibronectin-rich clot and form
new blood vessels. As the new wound tissue develops and

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the collagen scar forms, the blood vessels first mature and then regress (Clark 1996a). In contrast, during many pathologic processes and neoplastic diseases, new blood vessel growth proceeds unabated (Folkman 1994). Current
5 evidence indicates that the degree of tumor neovascularization correlates with metastasis and poor clinical prognosis in a variety of human cancers, including melanoma and breast carcinoma. Neovascularization is controlled by a dynamic balance of
10 angiogenic stimulators and inhibitors. Angiogenesis requires not only up-regulation of stimulators of vessel growth, but also down-regulation of inhibitors of vessel growth (Iruela-Arispe and Dvorak 1997). Elucidating
15 angiogenesis in vivo and in vitro should provide potential new therapeutic modalities to promote neovascularization in cutaneous wounds, and to inhibit angiogenesis in tumors, such as melanoma.

Angiogenesis is a complex process that involves many
20 sequential steps including adhesion, invasion, migration, proliferation and capillary tube formation (Folkman 1994; Arnold and West 1991; Bischoff 1997). The extracellular matrix (ECM) is involved in almost every step of angiogenesis, and angiogenesis occurs within the
25 interstitial ECM.

In the most commonly used assays of in vitro angiogenesis, endothelial cells are cultured on or in a fibrin gel, collagen gel or commercially available laminin gel (MATRIGEL™). Following appropriate
30 stimulation by angiogenic factors, cells aggregate and then differentiate into capillary-like structures (Blair et al. 1997; Goto et al. 1993; Madri et al. 1988; Sankar et al. 1996). Although these systems represented a major step forward in angiogenesis research, the quantification

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of capillary-like sprouts in these systems is difficult and results are often variable. Of more fundamental concern, however, is whether such in vitro systems simulate vasculogenesis rather than angiogenesis (Risau
5 and Lemmon 1988). A promising in vitro model of sprout angiogenesis was reported by Nicosia and Ottinetti (1990). They embedded sections of rat aorta into a fibrin matrix. Over a period of several days, small tubes comprised of endothelial cells grew from the aorta
10 muscularis (Nicosia and Ottinetti 1990). This system, however, has two major disadvantages: the tissue is not of human origin; and, the endothelial cell number and condition within the aortic sections cannot be easily defined. Nehls and Drenckhahn (1995a and 1995b)
15 developed another system in which bovine pulmonary artery endothelial cells cultured on microcarrier beads were embedded into a bovine-derived fibrin gel to study angiogenesis. However, even in unstimulated conditions, many endothelial cells individually migrated off the
20 microcarrier beads and invaded the fibrin gel. Nevertheless, using a similar model, Koblizek et al. (1998) demonstrated that angiopoietin-1 induced angiogenesis of rat adrenal-cortex-derived endothelial cells.

25 A need continues to exist for reliable, reproducible in vitro angiogenesis assays, which can be used to identify angiogenic stimulators and inhibitors for use in, for example, wound healing and cancer therapy, respectively.

30

SUMMARY OF THE INVENTION

The subject invention addresses this need by providing a cellular matrix system which comprises: cells of a selected cell type disposed on microcarrier

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particles; and an in vitro defined matrix having the microcarrier particles disposed therein. The in vitro matrix simulates a natural environment in which the cells naturally reside. In one particular embodiment, the
5 system is a cellular angiogenesis system comprising endothelial cells disposed on microcarrier particles and an in vitro defined fibrin matrix having the microcarrier particles disposed therein.

The invention further provides a method of
10 determining the effect on cellular migration/angiogenesis of a compound or effector cell of interest. The method comprises: providing cells of a selected cell type on microcarrier particles which are disposed in an in vitro defined matrix, wherein the in vitro matrix simulates a
15 natural environment in which the cells naturally reside; determining migration/angiogenesis of the cells into the in vitro defined matrix; including a compound or effector cell of interest in the in vitro defined matrix; redetermining migration/angiogenesis of the cells into
20 the in vitro defined matrix which includes the compound or effector cell of interest; and determining the effect on cellular migration/angiogenesis of the compound or effector cell of interest. In one particular embodiment, the method is for determining the effect on angiogenesis
25 of a compound or effector cell of interest and comprises: providing endothelial cells, preferably human microvascular endothelial cells, on microcarrier particles which are disposed in an in vitro defined fibrin matrix; determining angiogenesis of the cells into
30 the in vitro defined fibrin matrix; including a compound or effector cell of interest in the in vitro defined fibrin matrix; redetermining angiogenesis of the cells into the in vitro defined fibrin matrix which includes the compound or effector cell of interest; and

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determining the effect on angiogenesis of the compound or effector cell of interest.

In accordance with the subject invention, matrix components and culture conditions are optimized to
5 eliminate baseline cell migration. The assay is relatively easy and reproducible. The entire angiogenic process, including invasion, migration, tube formation, branching and network formation, can be recorded by time-lapse video-capture and analyzed by computer. This model
10 is a very powerful tool for investigations of human angiogenesis and can be applied to study the role of ECM receptors in angiogenic morphogenesis.

The invention also provides a method of promoting angiogenesis in a tissue. The method comprises:
15 selecting a tissue in which angiogenesis is desired; and introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

20 These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Figure 1 shows a three-dimensional human
25 angiogenesis model for assaying cell invasion, migration and tube formation of human dermal microvascular endothelial cells (HDMEC). (Step I) HDMEC are cultured on the surface of microcarrier beads to generate EC-beads. (Step II) EC-beads are embedded in fibrin gel,
30 collagen gel or fibrin/collagen gel, with or without presence of angiogenesis factor;

Figure 2 shows the formation of angiogenic sprouts and capillary tube-like structures by HDMEC in fibrin stimulated by VEGF, a) In control fibrin gel without

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addition of angiogenic factors, no significant sprout formation occurred after 48 hr. b) In the presence of VEGF (100ng/ml), HDMEC formed angiogenic sprouts, and invaded and migrated into the fibrin gel within 48 hr. c) By 7 days, VEGF-stimulated HDMEC formed branching capillary tube-like structures in the fibrin gel. 100X magnification;

Figure 3 illustrates the presence of lumen in capillary tube-like structures as demonstrated by confocal microscopic analysis. A) Phase-contrast photomicrograph of typical capillary tube-like structure formed by HDMEC in fibrin with 100 ng/ml VEGF. B) Series of contiguous 1 micron thick tangential cross sections of the capillary tube-like structure, obtained by reflective confocal microscopy with computerized imaging, initially revealed the top of the tube (0-3 microns) and then the presence of a central lumen (8-19 microns) between two walls. 400X magnification;

Figure 4 illustrates that VEGF stimulated sprout angiogenesis of HDMEC in fibrin in a concentration-dependent manner. Without the addition of VEGF, little to no sprout angiogenesis was observed in fibrin gel. The addition of increasing amounts of VEGF from 10 to 100 ng/ml resulted in a concentration-dependent increase in the number of beads with sprouts. Each data point represents the mean \pm standard deviation of triplicate determinations. The experiment shown is representative of 4 independent experiments;

Figure 5 shows the formation of capillary arcades and networks by HDMEC in fibrin with 100 ng/ml VEGF for 7 days. a) Overview of a capillary network. b) Branching capillary structures appeared to be composed of a linear array of multiple endothelial cells. Overview (c) and higher magnification (d) of a typical capillary network

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illustrate that networks formed as a result of branching and fusion of capillary tubes. Magnification: a) 40X, b) 100X, c) 40X, d) 200X;

Figure 6 shows a comparison of the angiogenic response of HDMEC to VEGF and VEGF-C at 48 hr. VEGF (open bars) and VEGF-C (shaded bars) both stimulated sprout angiogenesis of HDMEC in fibrin in a concentration-dependent manner. However, the angiogenic effect of VEGF-C was markedly less than that of VEGF. Each bar represents the mean \pm standard deviation of triplicate determinations. The experiment shown is representative of 3 independent experiments;

Figure 7 shows a comparison of the angiogenic response of HDMEC to bFGF and PDGF-BB at 48 hr. bFGF (open bars) stimulated sprout angiogenesis of HDMEC in fibrin with maximal response at 30 ng/ml. The angiogenic effect declined at higher concentrations of bFGF. PDGF-BB (shaded bars) in concentrations ranging from 10 to 100 ng/ml did not induce significant sprout angiogenesis of HDMEC in fibrin. Each bar represents the mean \pm standard deviation of triplicate determinations. The experiment shown is representative of 3 independent experiments;

Figure 8 shows that angiogenic blood vessels in early granulation tissue mature and then regress as wound repair progresses. Porcine wounds at 5 days (a,b), 7 days (c,d), and 10 days (e,f), were stained with Masson trichrome (a,c,e) and antibody to laminin (b,d,f). At 5 days (a,b), the wound space is almost filled with granulation tissue (gt), rich in newly forming microvessels (b), and the neoepidermis (e arrow) is migrating to close the wound. At 7 days (c,d) the neoepidermis has completely formed, the granulation tissue has organized, and the neovessels have matured and assumed a vertical orientation (d). At 10 days (e,f)

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wound contraction is underway (cc) and blood vessel regression is apparent (f). The bright lines near the top of panels d and f represent laminin in the epidermal basement membrane. The brightly staining annular and
5 oval ring-like structures in panels (b,d,f) represent blood vessels. e arrow: migrating neoepidermis; gt: granulation tissue; cc: contracting collagen neomatrix. Bar = 1 mm in a,c,e; 0.25 mm in b,d,f;

Figure 9 shows that as angiogenic blood vessels
10 mature and then regress, collagen replaces fibrin in the extracellular matrix milieu of the wound. Granulation tissue at 5 days (A,D), 7 days (B,E), and 10 days (C,F) was stained with antibody to laminin (A,B,C) or with Masson trichrome (D,E,F). Note the gradually increasing
15 deposition of collagen (fibrillar material in D,E,F) as angiogenic blood vessels (A) mature and assume a vertical orientation (B) and then regress (C). Bar = 80 microns in A,B,C; 50 microns in D,E,F;

Figure 10 shows that fibrin gels (a,c) and collagen
20 gels (b,d) differentially regulated HDMEC response to VEGF at 48 hr. Without the addition of angiogenesis factors, little or no sprout formation, invasion or migration of HDMEC occurred either in fibrin gel (a) or in collagen gel (b). In the presence of VEGF (100 ng/ml)
25 HDMEC formed capillary sprouts which invaded and migrated into fibrin gel (c). In contrast, in collagen gel in the presence of VEGF (100ng/ml) HDMEC invaded and migrated into the gel individually without forming capillary sprouts (d). 200X magnification; and

30 Figure 11 shows that fibrin facilitated sprout angiogenesis of HDMEC in collagen induced by VEGF at 72 hr. VEGF (100ng/ml) stimulated HDMEC to invade and migrate into pure collagen gel individually without forming capillary sprouts (a). With the addition of 17%

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fibrin fibrils, by weight (b) or 29% fibrin fibrils, by weight (c) to collagen gel in the presence of VEGF, sprout angiogenesis occurred together with individual cell invasion and migration. VEGF induced sprout
5 angiogenesis of HDMEC in pure fibrin gel (d). 100X magnification.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides a cellular matrix
10 system which comprises: cells of a selected cell type disposed on microcarrier particles; and an in vitro defined matrix having the microcarrier particles disposed therein. The in vitro matrix simulates a natural environment in which the cells naturally reside. In one
15 particular embodiment, the system is a cellular angiogenesis system and comprises: endothelial cells disposed on microcarrier particles; and an in vitro defined fibrin matrix having the microcarrier particles disposed therein.

20 The invention further provides a method of determining the effect on cellular migration/angiogenesis of a compound or effector cell of interest. The method comprises: providing cells of a selected cell type on microcarrier particles which are disposed in an in vitro
25 defined matrix, wherein the in vitro defined matrix simulates a natural environment in which the cells naturally reside; determining migration/angiogenesis of the cells into the in vitro defined matrix; including a compound or effector cell of interest in the in vitro
30 defined matrix; redetermining migration/angiogenesis of the cells into the in vitro defined matrix which includes the compound or effector cell of interest; and determining the effect on cellular migration/angiogenesis of the compound or effector cell of interest. In one

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particular embodiment, the method is for determining the effect on angiogenesis of a compound or effector cell of interest and comprises: providing endothelial cells on microcarrier particles which are disposed in an in vitro defined fibrin matrix; determining angiogenesis of the cells into the in vitro defined fibrin matrix; including a compound or effector cell of interest in the in vitro defined fibrin matrix; redetermining angiogenesis of the cells into the in vitro defined fibrin matrix which includes the compound or effector cell of interest; and determining the effect on angiogenesis of the compound or effector cell of interest.

The invention also provides a method of promoting angiogenesis in a tissue. The method comprises: selecting a tissue in which angiogenesis is desired; and introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue.

This general understanding of the subject invention is described in further detail below.

As used herein, cellular migration refers to the movement of cell(s) from point A to point B. For example, in the process of wound healing fibroblasts and endothelial cells migrate from a collagenous stroma extracellular matrix into a fibrin extracellular matrix. As another example, tumor cells migrate within a host, for example, from a collagen matrix into a basement membrane extracellular matrix or from a basement membrane extracellular matrix into a collagen extracellular matrix. Embryonic and fetal cells also migrate during tissue morphogenesis from one matrix to another. Cell migrate through a basal lamina, as when white blood cells migrate across the vascular basal lamina into tissues in response to infection or injury, or when cancer cells migrate from their site of origin to distant organs via

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the bloodstream or lymphatic vessels, a process known as metastasis.

Cellular angiogenesis refers to the complex process of new blood vessel formation by endothelial cells that involves at least five steps: cell invasion and migration, and capillary-tube formation, branching and fusion to form networks. Junctions, branches, and fusions define tube extensions. A junction is defined as the location where one tube enters and two or more tubes exit. A branch is defined as a tube that has left a junction point but has not fused with another tube. A fusion is defined as a tube, either before or after a junction, that has fused with an adjacent tube.

A cellular matrix system refers to a system which can be used to study cellular migration or cellular angiogenesis.

The cellular matrix system of the subject invention comprises cells of a selected cell type disposed on microcarrier particles. A selected cell type refers to any cell type of interest for determination of cellular migration/angiogenesis properties of that selected cell type. For example, the angiogenesis properties of endothelial cells are of interest (such as cultured human dermal microvascular endothelial cells), or the migration properties of tumor cells are of interest.

The cells of a selected cell type are disposed on microcarrier particles. As microcarriers there may be used all microcarriers which consist of a biocompatible material (such as, e.g., collagen, fibrin, polymers from hyaluronic acid or hyaluronan esters (e.g. ethyl or benzyl esters) (Andreassi et al. 1991; Myers et al. 1995) (e.g. HYAFF-11™ from FIDIA Advanced Biopolymers, Abano T., Italy), fibrin binding polypeptides, dextran, gelatin, silicone, etc.) or of a suitable mixture of

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several biocompatible materials. There may be used, for example, Cytodex3® (crosslinked dextran matrix with a denatured collagen layer, 100-230 μm) (Pharmacia AB, Sweden) based on dextran Cellgen (Koken Co., Japan), a
5 reconstituted and crosslinked collagen from bovine skin, 200-500 μm , or Cultispher-G® (macroporous gelatin microcarrier) (Hyclone, Greiner, Germany) based on gelatin.

The size of the microcarriers is not critical.

10 Usually, however, the carriers have a diameter of 50 to 500 μm . Microcarriers of a diameter below 50 μm are so small that their surface is generally not sufficient for cell growth. Microcarriers of a size higher than 500 μm are so large that a high coverage with cells is generally
15 difficult to achieve. Furthermore, these particles are not optimal for transplantation into in vitro assays or tissue.

The microcarriers can be pre-coated in order to improve the adhesion of the cells and enhance the extent
20 of coverage of the microcarriers. For the coating, proteins of the extracellular matrix, such as fibronectin or collagen, or whole eukaryotic cells (e.g. fibroblasts incapable of proliferation) or components thereof (e.g. membrane fractions) may be used.

25 The microcarrier particles are disposed in an in vitro defined matrix. The matrix simulates a natural environment in which the selected cell type naturally resides. For example, in wound repair the cell may be a skin fibroblast or endothelial cell and its natural
30 environment is collagenous stroma. The skin fibroblast or endothelial cell, during wound repair, naturally migrates from the collagenous stroma into a fibrin clot which fills the wound. The in vitro matrix of the subject invention is chosen to simulate the collagenous

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stroma within which the skin fibroblasts or endothelial cells naturally reside. For example, the in vitro matrix can be a contracted collagen gel produced according to the methods described in U.S. Patent No. 5,935,850, 5 issued August 10, 1999, the contents of which are incorporated herein by reference.

As used herein, a matrix is an interdigitating polymer bundle which refers to a scaffold in the cell's external environment with which the cells may interact 10 via specific cell surface receptors. Such a matrix can be a naturally occurring extracellular matrix such as, for example, collagen, fibrin, fibronectin, or hyaluronic acid, or an artificial extracellular matrix. Artificial extracellular matrices are biocompatible and include, for 15 example, dextran polymers, polyvinyl chlorides, polyglycolic acids, polylactic acids, polylactic coglycolic acids, and silicon. A description of artificial (or synthetic) extracellular matrices is provided in Putnam and Mooney (1996).

20 In order to be useful for the study of the effects of a compound or effector cell of interest on cellular migration/angiogenesis, the in vitro matrix is "defined". As used herein, an in vitro "defined" matrix refers to a matrix having defined, i.e. known, components. For 25 example, purified fibrin gels substantially free of stimulators or inhibitors are "defined" matrices, as are such fibrin gels with precise amounts of other plasma components added thereto. A fibrin gel with such other components added thereto is encompassed by the term 30 "fibrin" matrix as used herein.

As also used herein, a cellular migration/angiogenesis stimulator is a compound or effector cell which stimulates cells of the selected cell type to migrate in or from the in vitro defined matrix (a

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cellular migration stimulator), or which stimulates cells of the selected cell type to form new blood vessels (angiogenesis) (a cellular angiogenesis stimulator). Stimulates refers to a statistically significant increase
5 in migration or angiogenesis. A cellular migration/angiogenesis inhibitor is a compound or effector cell which inhibits cells of the selected cell type from migrating in or from the in vitro defined matrix (a cellular migration inhibitor), or which
10 inhibits cells of the selected cell type from forming new blood vessels (angiogenesis) (a cellular angiogenesis inhibitor). Inhibits refers to a statistically significant decrease in migration or angiogenesis. Substantially free means that any stimulators or
15 inhibitors that may be present are present in such an amount that negligible or statistically insignificant increases or decreases in the amount of migration/angiogenesis occur in the presence of such an amount of the stimulators or inhibitors.

20 In one embodiment of the subject invention especially useful for the study of angiogenesis, the cells are cultured human dermal microvascular endothelial cells disposed on microcarrier beads in a human derived defined fibrin matrix.

25 In alternate embodiments where one wishes to examine the cellular migration/angiogenesis of the cells of the selected cell type through the in vitro defined matrix toward another cell type, the cellular migration/angiogenesis system can further comprise cells
30 of a different selected cell type overlaid by or admixed with the in vitro defined matrix. "Different" refers to cells of a type distinguishable from the cells of the selected cell type (or detectable separate from). For example, one may wish to examine the formation of new

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blood vessels by endothelial cells induced by tumor cells. In one embodiment, this can be done by providing a layer or aggregate of tumor cells and having the in vitro defined matrix overlying the layer or aggregate of
5 cells (the in vitro defined matrix has the microcarrier particles disposed therein which have the endothelial cells disposed thereon). In another embodiment, this can be done by providing a droplet of tumor cells and having the in vitro defined matrix overlying the droplet (the
10 droplet may be an agarose droplet, for example, or may be the same material as the in vitro defined matrix or a different defined matrix).

Having thus described the cellular matrix system according to the subject invention, the system can be
15 used to determine the effect on cellular migration/angiogenesis of a compound or effector cell of interest. A compound of interest refers to a biological response modifier. Examples of such biological response modifiers include, but are not limited to, chemotactic
20 factors, growth factors, cytokines, autocoids, and prostanoids. An effector cell of interest refers to a cell that causes an effect of interest.

If the in vitro defined matrix overlies cells of a different selected cell type (such as in the embodiment
25 described above where one wishes to examine the formation of new blood vessels by endothelial cells toward or induced by tumor cells), then one would detect the migration/angiogenesis of the cells of the selected cell type toward the cells of the different selected cell
30 type.

It should be readily apparent to those skilled in the art that the migration/angiogenesis of cells can only be monitored or studied if the cells are detectable. This can be accomplished in several ways. In one

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embodiment the cells may be visualized with a light microscope. Alternatively, the cells can be labeled with a detectable marker. Such detectable markers are known in the art, and include, for example, radioactive labels, 5 fluorescent labels, vital dyes (these non-toxic dyes stain living cells), and labels added by molecular manipulation (such as the β gal gene). The migration/angiogenesis of the labeled cells can thus be monitored.

10 Cells of a selected cell type are disposed on microcarrier particles using methods known in the art (one such method is further detailed below). These "coated" microcarrier particles are then disposed in the in vitro defined matrix also using methods known in the 15 art (one such method is further detailed below).

A fibrin matrix which is substantially free of fibronectin as well as stimulators and inhibitors of cell migration/angiogenesis can be made in accordance with U.S. Patent Application No. 09/500,512, filed February 9, 20 2000, the contents of which are incorporated herein by reference. This method is further detailed in the Materials and Methods section below.

A compound of interest can be introduced into the in vitro defined matrix using methods known in the art (one 25 such method is further detailed below).

This entire cellular matrix system and method can be automated. In such an automated system, cultured endothelial cells are transferred to microcarrier beads robotically. Appropriate concentrations of fibrinogen or 30 other gelable material (as the in vitro matrix) are added to multiwell plates robotically. The endothelial cells on beads at a desired density are transferred to the wells robotically. The multiwell plates are then robotically handled in a manner to allow gelation of the

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in vitro matrix. Medium containing stimulatory or inhibitory factors to be tested is added to the plates robotically. Plates are incubated in the desired environment for the desired time robotically. Using a
5 microscope with a motorized stage and that is coupled to a camera or video (that is interfaced with a computer that is configured with image digitizer software), images of the beads within each well are identified, recorded and transferred to a file robotically. Alternatively,
10 plates are processed robotically to fix and stain endothelial cells migrating out from the beads. Using the same imaging system, cells in each well are identified, recorded and transferred to a file robotically. Using image analyzing software the number
15 of cell processes or tubes extending from the beads is quantified and the extension length determined.

The subject invention also provides a method of promoting angiogenesis in a tissue. The method comprises selecting a tissue in which angiogenesis is desired, and
20 introducing cultured endothelial cells into the tissue to promote angiogenesis in the tissue. This method is described more fully below. The cultured endothelial cells can be provided as an endothelial cell aggregate or as endothelial cells disposed on microcarrier particles.
25 As used herein, a tissue could be an in vitro tissue (for example, for research purposes, or for transplantation or implantation or grafting or other use in vivo), or an in vivo tissue (for example, for treatment purposes). The tissue can be naturally occurring tissue or an artificial
30 tissue. A tissue sample of particular interest would be wound tissue (such as artificial tissue for wounds). As used herein, a wound refers to both acute and chronic dermal wounds including, for example, surgical incisional wounds, traumatic wounds, cancer extirpations, radiation

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wounds, venous leg ulcers, diabetic ulcers, pressure ulcers, and burn wounds.

MATERIALS AND METHODS

5 Materials

Gelatin-coated microcarrier beads (Cytodex-3) were purchased from Pharmacia (Uppsala, Sweden). Type 1 collagen was obtained from Collagen Biomaterials (Palo Alto, CA). Dimethyl dichlorosilane, aprotinin, dibutyl 10 cyclic AMP, hydrocortisone, trypsin, soybean trypsin inhibitor, and EDTA, were obtained from Sigma Chemical Co. (St. Louis, MO). Endothelial cell basal medium (EBM), bovine brain extract, and epidermal growth factor were obtained from Clonetics Corp. (San Diego, CA). 15 Normal human serum was obtained from BioWhittaker, Inc. (Walkersville, MD). Vascular endothelial cell growth factor (VEGF) was purchased from Peprotech (Rocky Hill, NJ). Basic fibroblast growth factor (bFGF) was obtained from Scios Nova, Inc. (Mountainview, CA). Platelet 20 derived growth factor-BB (PDGF-BB) was kindly provided by Charles Hart (Zymogenetics, Seattle, WA). VEGF-C was kindly provided by Kari Alitalo (Cao et al. 1998). Human thrombin was obtained from Calbiochem (San Diego, CA).

Culture and characterization of HDMEC

25 HDMEC were isolated from human neonatal foreskin. Briefly, after initial harvest from minced trypsinized human foreskins, microvascular endothelial cells were further purified on a Percoll density gradient. HDMEC were cultured on collagen type 1 coated tissue culture 30 flasks in EGM (endothelial cell growth medium) consisting of EBM supplemented with 10 ng/ml epidermal growth factor, 0.4% bovine brain extract, 17.5 microg/ml dibutyl cyclic AMP, and 1 microg/ml hydrocortisone in the presence of 30% normal human serum. Endothelial cell

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cultures were characterized and determined to be >99% pure on the basis of formation of typical cobblestone monolayers in culture, positive immunostaining for factor VIII-related antigen, and selective uptake of acetylated
5 low density lipoprotein. All experiments were done with HDMEC below passage 10.

Preparation of HDMEC-loaded microcarrier beads (EC-beads)

Gelatin-coated cytodex-3 microcarrier beads were prepared as described by the manufacturer. Approximately
10 80,000 sterile microcarrier beads were washed, resuspended in EGM, and added to approximately 4.5 million HDMEC. The beads and cells were mixed by gentle swirling, incubated at 37°C for 6 hr, and then rotated for 24-36 hr on an orbital mixer in a 37°C oven to
15 generate endothelial cell-loaded microcarrier beads (EC-beads).

Cell migration and capillary sprout formation in fibrin gels and type I collagen gels

A microcarrier in vitro angiogenesis assay
20 previously designed to investigate bovine pulmonary artery endothelial cell angiogenic behavior in bovine fibrin gels (Nehls and Drenckhahn 1995a; Nehls and Drenckhahn 1995b) was modified for the study of human microvascular endothelial cell angiogenesis (Figure 1).
25 Briefly, human fibrogen, isolated as previously described (Mosesson and Finlayson 1963a and 1963b), was dissolved in M199 medium at a concentration of 1.5 mg/ml and sterilized by filtering through a 0.22 μ m filter. Collagen solution was prepared by dissolving sterile type
30 1 collagen in M199 medium at a concentration of 1 mg/ml. In certain experiments, growth factors, such as VEGF, VEGF-C, bFGF or PDGF-BB, were added to the fibrinogen and collagen solutions. In certain experiments, an aliquot of fibrinogen solution was added to collagen solution

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prior to gelation. About 500 EC-beads were then added to the ECM protein solutions, followed by the addition of 0.5 U/ml human thrombin. A 0.3 ml aliquot of each suspension was immediately added to appropriate wells of a 24-well tissue culture plate. After gelation, 1 ml of fresh assay medium (EBM supplemented with 20% normal human serum) was added to each well. The HDMEC angiogenic response was monitored visually and recorded by video image capture. Specifically, capillary sprout formation was observed and recorded with a Nikon Diaphot-TMD inverted microscope (Nikon, Inc., Melville, NY), equipped with a incubator housing with a Nikon NP-2 thermostat and Sheldon #2004 carbon dioxide flow mixer. The microscope was directly interfaced to a video system consisting of a Dage-MTI CCD-72S video camera and Sony 12" PVM-122 video monitor linked to a Macintosh G3 computer. The images were captured at various magnifications using Adobe Photoshop. The effect of angiogenic factors on sprout angiogenesis was quantified visually by determining the number and percent of EC-beads with capillary sprouts. One to two hundred beads (five random low power fields) in each of triplicate wells were counted for each experimental condition. All experiments were repeated at least three times.

25 Porcine Cutaneous Wounds and Immunofluorescence Staining

Porcine cutaneous wounds were harvested at various times and then immunoprobed for expression of integrin receptors as previously described (Clark et al. 1996b). Briefly, full-thickness wounds were made with an 8-mm punch on the backs of White Yorkshire pigs and harvested at the times indicated. Specimens were bisected; one half was fixed in formalin and stained with Masson trichrome, the other half was frozen in liquid nitrogen for immunofluorescence studies. Anti-laminin antibodies

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(Gibco BRL) that were conjugated with biotin were used to identify wound vasculature. All antibodies were used at dilutions that gave maximal specific fluorescence and minimal background fluorescence on frozen tissue

5 specimens. Bound antibody was detected by the avidin-biotin-complex (ABC) technique. Stained specimens were observed and photographed using a Nikon Microphot FXA epifluorescence microscope equipped with a Nikon FX-35DX 35 mm camera.

10 Confocal microscopy

Confocal microscopy was done at the University Microscopy Imaging Center, Health Sciences Center, SUNY at Stony Brook, to confirm that sprouts emanating from the EC-beads formed tubes. For these studies EC-beads
15 (150) were suspended in a three dimensional fibrin gel with VEGF (100ng/ml) in a 4-well Lab-Tek Chambered Coverglass and incubated for 7 days. Samples were washed in 2XPBS and then fixed in 2% paraformaldehyde. Images were sequentially obtained (1 micron contiguous
20 tangential cross sections) using a Noran laser scanning confocal system (Odyssey; Noran Instruments, Inc., Middleton, WI) attached to a Nikon Diaphot-TMD microscope. A Silicon Graphics Iris workstation was used for processing digitized micrographs and assembling
25 three-dimensional renditions from confocal images using VoxelView software (Voxelview, Vital Images).

Detailed Reagents.

Solution 1: Endothelial Cell Culture Media (ECCM)

Add to 500 ml EBM: Endothelial Basal Media
30 (Clonetics, San Diego):

75 ml denatured normal human serum (NHS)
(Biowhittaker, Walkersville)

25 ml of 0.35 mg/ml dibutyryl cyclic AMP in EBM
(Sigma, St. Louis)

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2 ml of Bovine Brain Extract (Clonetics)
500 μ l of 1 mg/ml hydrocortisone in ethanol
(Sigma, St. Louis)

500 μ l of 10 μ M mouse EGF (epidermal growth
5 factor) in PBS (Clonetics)

Solution 2: Pretreated Microcarrier Beads

Suspend 1 g (4 million) sterile, gelatin-coated
microcarrier beads (Cytodex-3, Pharmacia, Uppsala,
Sweden) in 50 ml sterile phosphate-buffered saline
10 (PBS, pH 7.4) and incubate at 37°C for 5 hr.

Aspirate the supernatant. Add 50 ml PBS. After
about 10 min of sedimentation, again aspirate the
supernatant, and wash once more with PBS.

Resuspend the beads in 50ml PBS and autoclave
15 at 115°C, 15 psi for 15 min.

Allow the beads to settle. Aspirate the
supernatant, resuspend the beads in 50 ml Solution 1
(ECCM), and store at 4°C for at least 2 days, but
for no longer than one month, prior to use in
20 experiments.

Solution 3: Cell Harvest Solution

Trypsin 0.025% / EDTA 0.01% solution (1 x)
(Clonetics)

Solution 4: Extracellular Matrix Solution (EMS)

25 95% clottable fibrinogen (prepared as described
below) dissolved in EBM (Clonetics) at 1 mg/ml,
sterilized by filtration through 0.2 μ m filter,
aliquotted and stored at -70°C.

Solution 5: Clotting Enzyme Solution

30 0.2 U/ μ l human thrombin (Calbiochem, San Diego), in
sterile water

Solution 6: Angiogenesis Culture Media (ACM)

Add to 200 ml of EBM:

40 ml normal human serum (NHS)

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240 μ l Aprotinin (Sigma, St. Louis) (10 μ g/ μ l dissolved in PBS)

Detailed Culture of human microvascular endothelial cells on microcarrier beads.

- 5 1. Add 15 ml of Solution 1 (ECCM) and 1 ml Solution 2 (beads) to a sterile 50 ml polypropylene conical tube. Stand tube in the hood to sediment the beads. Aspirate the supernatant about 20 min later.
- 10 2. Wash one tissue Culture flask (165 cm²) of 90% confluent human microvascular endothelial cells (4-5 million cells) 3 times with PBS. Add 2 ml of Solution 3 (Trypsin/EDTA). When cells round up/detach, add 8 ml Solution 1 (ECCM) to neutralize Solution 3 (Trypsin/EDTA). Transfer the cell
15 suspension to a 15 ml sterile polypropylene conical tube and centrifuge at 1,000 rpm for 8 min. Aspirate the supernatant and resuspend the cell pellet in 6 ml of Solution 1 (ECCM).
- 20 3. Add the 6 ml of cell solution to the 50 ml tube containing the prepared beads.
4. Gently mix the beads and the cells by swirling the tube. Loosen the cap and incubate the tube in the CO₂ incubator at 37°C. For the initial 6 hr, swirl the tube once every 1.5 hr (4 times).
- 25 5. After incubation for 6 hr, add an additional 25 ml of Solution 1 (ECCM) pre-warmed to 37°C and seal the cap tightly. Load the tube onto the orbital mixer in a 37°C oven, and rotate the tube on the mixer for 24-36 hr. This produces endothelial cell-loaded
30 microcarrier beads (EC-beads).

Note: Before using EC-beads in experiments, quantitate the EC-beads and examine them for quality control. Take a 48-well or 24-well plate, add 500 μ l PBS and add 100 μ l EC-bead solution. Observe whether the bead surfaces are

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covered by relatively confluent microvascular endothelial cells. Count and calculate the concentration of EC-beads.

Detailed Angiogenesis Assay.

1. Thaw an aliquot of Solution 4 at 37°C for 10-15 min.
- 5 2. For each condition to be tested, add 1 ml of Solution 4, with or without the addition of an appropriate concentration of proangiogenic or antiangiogenic factor, to a 1.5 ml sterile Eppendorf tube, and vortex.
- 10 3. Five hundred freshly prepared EC-beads will be needed for each condition to be tested. Add the appropriate number of EC-beads to a 50 ml sterile polypropylene conical tube. Wash twice by gentle sedimentation in 10 ml aliquots of EBM. After
15 aspirating the second wash supernatant, add M μ l of EBM (where $M=50 \times [\text{number of conditions}+2]$) to resuspend the EC-beads.
4. Add 50 μ l of EC-bead preparation to each Eppendorf tube (step 2).
- 20 5. Add 2.5 μ l of Solution 5 (thrombin) to each tube of EC-bead preparation, and immediately mix by pipetting up and down gently three times.
6. For each condition to be tested, add 300 μ l of the appropriate EC-bead preparation to each of
25 triplicate wells in a 24-well tissue culture plate.
7. Allow the plate to remain at room temperature for 20 min to permit proper polymerization of the fibrin gels.
8. Add 1 ml of Solution 6 to each well and incubate the
30 plate at 37°C, 5% CO₂.

In the presence of angiogenic factors, microvascular endothelial cells will invade the fibrin gel and form capillary sprouts within 48-72 hrs, and form capillary networks within 5 days. Negative control (no added

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factor) and positive control (VEGF, 100 ng/ml) should be included in every experiment.

EXAMPLE I

5 Preparation of Fibrinogen

The following discussion of the preparation of fibrinogen is taken from co-pending, co-assigned U.S. Patent Application Serial No. 09/500,512, filed February 9, 2000, the contents of which are hereby incorporated by
10 reference.

The plasma employed in the preparation of fibrinogen is collected by conventional methods and, in practice, can be from blood of a single individual, or, alternatively, it can be pooled from multiple
15 individuals.

As indicated below, fibrinogen is isolated from the plasma by precipitation. In particular, the method of precipitation is achieved with glycine and is carried out in a number of steps.

20 As a first step, the plasma is precipitated with glycine to produce a precipitate and a supernatant in a manner known to those of ordinary skill in the art. A preferred method is described in Galanakis (1995). Preferably, precipitation is carried out by adding
25 glycine to the plasma in an amount such that the final concentration of glycine in the plasma/glycine mixture is from about 1.0 to about 2.1 M. Preferably, the glycine is added as dry glycine to the mixture. Once the addition is complete, precipitation is allowed to proceed
30 during incubation. Incubation proceeds at temperatures below room temperature (e.g., refrigeration temperatures), preferably from between about 2 °C and about 7 °C, more preferably about 5 °C. Incubation occurs for from about 30 minutes to about 12 hours,

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preferably about 1 hour, until the precipitate is formed. In practice, it is most convenient to conduct the precipitation by placing the plasma/glycine mixture in a standard refrigerator (i.e., at about 5 °C). After
5 incubation, a precipitate and a supernatant are produced, which can be separated by conventional methods, such as decanting or, preferably, centrifuging at temperatures from between about 2 °C and about 7 °C, preferably about 5 °C. The precipitate will contain
10 about 90% of the fibrinogen from the plasma. The purity of the fibrinogen is above 50%.

If a preparation having high purity content is desired, fibrinogen is further isolated from the precipitate of the first step. A high purity content
15 fibrinogen is defined as fibrinogen having a purity content of about or above 99%.

A second step is used to isolate fibrinogen from the precipitate of the first step. As used herein, second step generally refers to the process of adding buffer to
20 a precipitate to produce a mixture, adding glycine to the mixture to produce a precipitate and a supernatant, and separating the precipitate and supernatant. This second step can be repeated as many times as desired.

Typically, the precipitate produced in the first
25 step is dissolved in a suitable buffer to produce a solution. Preferably, the buffer has a pH of from about 6 to about 8, preferably from about 6.2 to about 7.6, most preferably about 6.4. One suitable buffer for carrying out this process contains about 150 mM of sodium
30 chloride, about 10 mM sodium phosphate, and 100 mM epsilon-aminocaproic acid in water, preferably in sterile water suitable for injection. Other buffers suitable in the present invention include 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The amount of buffer employed to effect

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the dissolution is preferably from about 30% to about 40% of the volume of the original plasma used in the first step. That is, if the precipitate is precipitated in the first step from plasma having a volume of V, the buffer
5 used in this second step preferably has a volume of from about 0.3 V to about 0.4 V (i.e., between about 3/10's of V to about 4/10's of V). More preferably, the volume of buffer employed to effect the dissolution of the precipitate is about 35% of the volume of the plasma used
10 in the first step.

Glycine, typically dry (as described above), and at a suitable concentration is then added to the resulting solution in an amount such that the final concentration of glycine in the resulting mixture is from about 1.7 to
15 about 2.2 M and, more preferably, about 2.1 M. The resulting mixture is incubated, preferably at a temperature of from between about 2 °C to about 7 °C, most preferably at about 5 °C, for from about 30 minutes to about 2 hours, preferably for about one hour.

20 As a result, a precipitate and supernatant form, which are separated, preferably by centrifugation at from between about 2 °C to about 7 °C, most preferably at about 5 °C.

This second step is advantageously repeated several
25 times. Preferably, the second step is repeated at least twice. The precipitate at the end of the second step will contain about 60% of the fibrinogen from the plasma. The fibrinogen is at a purity of about 90%.

To produce fibrinogen having a low purity content,
30 fibrinogen can be additionally or alternatively isolated from the supernatant (instead of the precipitate) of the original plasma/glycine mixture of the first step in a manner similar to that described above. As used herein, low purity fibrinogen means fibrinogen having a purity

content of about or above 95%, but below 99%. The low purity fibrinogen is produced by adding glycine, typically dry and at a suitable concentration, to the supernatant of the first step to produce a mixture. The
5 resulting mixture is incubated, preferably at refrigerator temperatures, for about 1 hour. As a result, a precipitate and supernatant form, which are separated, preferably by centrifugation at refrigerator temperatures. The precipitate can then be dissolved in
10 an appropriate buffer (e.g., the ones described above), glycine added (preferably to a final glycine concentration of about 2.1 M), the mixture incubated, and the resulting precipitate separated. This process can be repeated several times.

15 Irrespective of whether the fibrinogen is isolated as a precipitate from the precipitate or supernatant or both of the original plasma/glycine mixture described above, the precipitate can be advantageously further treated to purify the fibrinogen. Typically, the further
20 treatment includes dissolving the precipitate resulting from the glycine precipitation(s) described above in an appropriate buffer (e.g., as described above) to produce a solution where the precipitate is present in a volume of 1/2 to 1/3 of the original plasma, with 1/3 being
25 especially preferred, and precipitating this solution. Preferably, the precipitation is achieved by adding a compound such as ammonium sulfate to the solution. Typically, the ammonium sulfate is added as a saturated solution, and the amount of ammonium sulfate in the
30 solution is about 25 percent of its saturation level. The resulting solution is redissolved in a suitable buffer (as described above) and reprecipitated, preferably with dialysis in 0.3 M NaCl.

Using the method of the present invention, a precipitate fraction of fibrinogen with a purity of greater than 95% (as ascertained by SDS-polyacrylamide gel electrophoresis ("SDS-PAGE")) is obtained. When high
5 purity fibrinogen is desired, using the method of the present invention fibrinogen having a purity of about or above 99% is obtained.

In one embodiment, the preparation of fibrinogen is as follows.

10 *Fibrinogen Preparations from Precipitate*

The fibrinogen preparations of the present invention were prepared from a 1M glycine precipitation formed in the cold (4°C) that had been discarded in a previously described procedure (Galanakis 1995). In particular,
15 glycine was dissolved in pooled human plasma to attain 1 M final concentration and the mixture was allowed to stand on ice for at least one hour. By subsequent centrifugation at 5°C, two fractions were obtained: the precipitate and the supernatant. The precipitate obtained
20 from plasma containing 1 M glycine, 5°C, was redissolved (pH 6.4) and re-precipitated twice with 2.1 M glycine, 5°C. The precipitate was then dissolved, precipitated with $(\text{NH}_4)_2\text{SO}_4$ to 25% saturation, redissolved and exhaustively dialyzed vs. 0.3M NaCl. The final product
25 was stored at -80°C. Greater than 95% purity was ascertained by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels also disclosed the expected doublet of fibrinogen bands I and II indicating that these bands did not differ from those of fibrinogen in unfractionated
30 plasma.

Fibrinogen Preparations from Supernatant

The following preparations were purified from the supernatant produced during precipitation of the fibrinogen precipitate described above.

- 30 -

Sample 1 was isolated as previously described (Galanakis 1995). It was the purest fibrinogen isolate. It had virtually all its alpha chains intact but lacked molecules with gamma chains that have an extended carboxy
5 terminal which constitute approximately 15% of the fibrinogen in plasma. The extended gamma chains are from a splicing mRNA variant. It extends the gamma chain by deleting the last two amino acids of the regular gamma chain and extending it with a 20 amino acid segment.

10 Sample 2 was isolated by a modification (Galanakis 1995) of the procedure of Mosesson and Sherry (1966). Sample 2 was a highly pure fibrinogen isolate, but less pure than Sample 1, in that a minute contaminant of Factor XIII was detectable by biologic activity. It also
15 had virtually all of its alpha chains intact and contained molecules with both extended and non-extended gamma chains.

Sample 3 was the same as Sample 2, but was enriched with soluble fibrin. Fibrin monomer was prepared as
20 described in Galanakis et al. (1987). An amount of fibrin monomer (from a stock solution of 20 to 30 mg/ml, pH 4.5) was added to exceed 10% (mg/mg) of fibrinogen in solution, allowed to equilibrate at 37°C and any clot that formed was removed. The resulting fibrinogen
25 solution was termed fibrin-saturated and used. Care was taken to ascertain that the pH of the fibrinogen solution remained above 6 in storage and during clotting.

Sample 4 was isolated by a modification (Galanakis 1995) of the procedure of Mosesson and Sherry (1966).
30 Sample 4 had a purity similar to that of Sample 3, but contained a major population of molecules (approx. 20 to 30%) which were clottable but had degraded alpha chains.

Sample 5 was isolated by a modification (Galanakis 1995) of the procedure of Mosesson and Sherry (1966).

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Sample 5 was similar to Sample 4 in structure but less pure in that trace, but detectable, amounts of fibronectin and factor XIII were present.

Sample 6 was similar in all respects to Sample 5, with the exception of being enriched with soluble fibrin. Fibrin monomer was prepared and added to Sample 5 as described above for Sample 3.

Sample 7 was isolated from the supernatant produced in Sample 1 above. It contained only minor amounts of fibrinogen, but was rich in plasma lipids.

Empirical observations have shown that a fibroblast migration enhancement (FME) property is present in fibrinogen preparations of relatively low purity and this activity is stable on freeze thawing of such preparations. Conversely, fibrinogen preparations that are of the highest possible purity, such as fibrinogen fraction I-4, DEAEc peak 1 fraction, and others (produced by methods known to those skilled in the art) either possess low or no activity. Of particular use is fraction I-4, which can be prepared in bulk and whose moderate FME activity progressively decreases on repeat freeze thawing, thus enabling its use as a negative control. In the discussion below, positive and negative controls are termed C1 and C2, respectively. Fibrinogen isolates with high activity are isolated from plasma by procedures that yield two kinds of active isolates, one enriched with soluble fibrin and the other lacking fibrin enrichment. The procedures also yield a lipid or lipoprotein rich (L) component which is enriched in FME activity.

Fibrin-rich fibrinogen preparation.

Glycine is dissolved in plasma to 1 M (or 1 molar) concentration and allowed to stand at 4° C overnight. The precipitate formed is dissolved in phosphate buffered

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saline, pH 6.4, subjected to reprecipitation with 2.1 M Glycine at 4° C, and this step is repeated. An additional precipitation step is performed with either 2.1 M Gly or 25% saturated Ammonium Sulfate. The
5 fibrinogen isolate thus obtained is dialyzed vs 0.3 M NaCl at 4° C and termed 2F. As shown below, fibrinogen 2F may be further separated into sub-fractions. A large amount of insoluble fibrin gel forms during dialysis, and this is removed by centrifugation as described below.

10 *Fibrinogen low in fibrin content preparation.*

An isolate is obtained from the plasma supernatant of the initial 1 M Gly step detailed in the paragraph above. For this purpose, additional Glycine is added and dissolved to achieve 2.1 M Gly concentration and the
15 precipitate obtained at 4° C is subjected to the same precipitation steps described above. The final isolate is dialyzed as above and termed 2H. As shown below, fibrinogen 2H may be further separated into sub-fractions. During dialysis a small amount of insoluble
20 fibrin forms and is removed by centrifugation as described in the paragraph below.

Further sub-fractionation to obtain and characterize sub-fractions.

Each of the fractions, 2F and 2H, is subjected to
25 centrifugation using at least 4000 XG for 30 or more minutes at 4° C. The resulting three sub-fractions are illustrated in Fig. 1. One sub-fraction is a lipid or lipoprotein component which contains insoluble material floating at the top of the solution. This subfraction is
30 termed 2FL (from the 2F fibrin rich fraction) or 2HL (from the 2H low fibrin content fraction). Use of a spatula or other implement permits harvesting these sub-fractions. The second sub-fraction is the bulk of isolated fibrinogen and is referred to herein as 2F sub-

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fraction (fibrin rich fibrinogen) or 2H sub-fraction (low fibrin content fibrinogen). The third sub-fraction is a pellet at the bottom of the centrifuged solution, which consists of insoluble fibrin gel and is discarded.

5 During the harvest and testing of the lipid and fibrinogen sub-fractions certain characteristics emerge. One is that fibrinogen 2H invariably contains major amounts of sub-fraction 2HL, and fibrinogen 2F, by contrast, tends to contain lower amounts of sub-fraction
10 2FL. Such differences are demonstrable by dissolving the material and measuring its turbidity. Further, some 2FL sub-fractions are of substantial amounts while others are of very small amounts compared to those in the 2HL counterparts from the same starting plasma. A
15 constellation of characteristics of the L isolates is their self-evident low density and coalescence into insoluble floating sheets or particles following centrifugation, their yellowish-white and opaque appearance on visual inspection (particularly marked in
20 2HL), their capacity to be readily dispersed and re-dissolved in fibrinogen containing buffers, and their high turbidity when re-dissolved and assessed spectrophotometrically.

To compare the FME activity of 2HL or 2FL with their
25 2H or 2F counterparts, fibrin is formed in the assay from the same lot. Comparison can also be made with the same parent (or uncentrifuged fibrinogen preparation). In order to test the L moiety of 2FL or 2HL alone, fibrinogen can be removed so that the sub-fraction can be
30 tested without its parent fibrinogen. To remove its fibrinogen, ammonium sulfate is added to 25% saturation and the precipitate is discarded. The floating insoluble material on the top of the solution is then harvested and dialyzed.

An alternate method to sub-fractionate 2F or 2H isolates.

The fractions can be sub-fractionated by subjecting them to size exclusion chromatography, as shown in Fig.

2. This enables removal of most of the lipid and of the
5 soluble fibrin components, so that each such component
can be tested. Absorbance values of the eluting
fractions are obtained at 280 nm and at 350 nm.

Absorbance at 280 nm reflects the presence of protein and
that at 350 nm reflects light scattering and, thus, the
10 opacity caused by the lipid content of the fibrinogen
solution. This chromatographic procedure results in an
early elution peak, labeled peak I. Fractions containing
this peak appear white-opaque, show high absorbance
values at 350 nm as expected, and show the presence of
15 protein by their absorbance values at 280 nm. When
dialyzed against water and freeze dried, this peak is
insoluble in buffer but can be resolubilized at least in
part in fibrinogen or other protein solutions. The
second peak, labeled peak II, consists of fibrinogen and
20 soluble fibrin. This peak shows absorbance at 280 nm but
little or negligible absorbance at 350 nm, as shown, and
constitutes the bulk of protein applied to the column.

*Fibroblast enhancing (FME) activity: 2F, 2H and non-
chromatographic subfractions.*

25 Fractions 2F and 2H have ample activity, with 2H
showing moderately higher activity than 2F. Similarly,
this activity remains high, as shown in Fig. 3, in both
the L rich subfractions (2FL, 2HL) and fibrin rich sub-
fractions (2H, 2F). Moreover, these sub-fractions, 2FL
30 and 2HL, show similarly high activity (See Fig. 3 insert)
and this activity remains when fibrinogen is removed from
2HL and 2FL (not shown). Because insoluble lipid in 2HL
may induce formation of an abnormal fibrin matrix, care
is taken to avoid insoluble lipid aggregates when using

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2HL. This is not the case with 2FL, which contains a much lower lipid content and lacks such large insoluble aggregates.

Fibroblast enhancing (FME) activity: Chromatographic

5 *subfractions.*

Tested as outlined above, both peaks (shown in Fig. 2) possess substantial fibroblast migration enhancing activity. The fibroblast migration activity of the lipoprotein (L) peak of Fig. 2 is shown in the Fig. 4.

10 This is consistent with the results from the post-centrifugation sub-fractions described above, indicating that the activity is present both in the L rich and the fibrinogen (L poor) peaks. Considering the small amount of L sub-fraction required to demonstrate the activity,
15 this implies the lipid in these isolates contains relatively higher activity than does fibrinogen per se. That is to say, the activity is more lipophilic than fibrinogenophilic. Moreover, when fibrin-rich fibrinogen was isolated from the ascending limb of the chromatogram
20 it too displayed substantial activity, not shown, consistent with the fact that fraction 2F is fibrin rich and shows activity comparable to 2H.

Summary and conclusions.

The fibroblast migration enhancement activity of
25 fibrinogen isolated by the above procedures is associated with three components or sub-fractions: fibrin rich fibrinogen, lipid rich fibrinogen, and fibrinogen not enriched with either fibrin or lipid. Although lipid rich fibrinogen (2FL or 2HL) displays somewhat higher
30 activity, this activity remained when the lipid component was rendered free of fibrinogen by chromatography or other means. Moreover, fibrin rich fibrinogen which is also rendered lipid poor clearly retains its high activity. In another set of observations (data not

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shown), this activity is progressively lost by freeze thawing of I-4, and absent in DEAEc pure fibrinogen. Taken together, these results imply a non-fibrinogen and possibly non-lipid hydrophobic agent whose FME activity
5 remains stable in storage of fibrinogen/lipid mixtures. This explains its stability in fibrinogen isolates of relatively low purity (i.e. $\geq 95\%$ by protein measurements), and enables potential use of such preparations in situations where enhancement of wound
10 healing is clinically important. Also, in order for fibrinogen isolates to possess the highest FME activity they need be enriched with substantial amounts of a lipid component that co-isolates with them from normal plasma and results also in stability of this activity when
15 fibrinogen is stored frozen and re-frozen. What is more, the lipid component can be isolated and re-introduced into fibrinogen of high purity or any other fibrinogen isolate for the purpose of further enriching its FME activity. This discovery makes it possible to monitor
20 the amounts of this lipid component in any fibrinogen and/or soluble fibrin preparations.

EXAMPLE II

Characterization of in vitro angiogenesis of HDMEC in 25 three-dimensional fibrin

To study the regulation of human angiogenesis, a relatively simple, reproducible, three-dimensional in vitro angiogenesis assay for HDMEC was established by modification of a previously described in vitro bovine
30 angiogenesis model (Nehls and Drenckhahn 1995a; Nehls and Drenckhahn 1995b) (Figure 1). In this assay, HDMEC cultured on the surface of microcarrier beads (EC-beads) are embedded in three-dimensional fibrin, collagen, or fibrin/collagen gels with or without the addition of an

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angiogenesis factor. When an angiogenesis factor was added to fibrin gel containing EC-beads, HDMEC formed capillary sprout-like structures, which projected from the surface of the EC-beads and invaded into the fibrin gel within 48 hr (Figure 2b). Note that the base of each sprout was in intimate contact with the surface of the EC-bead (Figure 2b). By 7 days the endothelial sprouts had elongated and in some cases formed branching capillary tube-like structures (Figure 2c). In contrast, without the addition of an angiogenesis factor, no significant HDMEC sprout formation occurred from the surface of EC-beads in fibrin gel, despite the presence of 20% normal human serum in the medium (Figure 2a).

To demonstrate whether capillary tube-like structures (Figure 3A) had lumina, reflective confocal microscopic analysis was used. Computer-assisted sectioning clearly revealed the presence of a lumen (Figure 3B) in the capillary-like structure shown in Figure 3A. As the tube was cut through by a series of 1 micron contiguous tangential cross sections, first the top and then the central lumen with two walls become visible (Figure 3B). Thus, critical steps of human angiogenesis, i.e. capillary sprout formation, migration and invasion of a 3-dimensional human ECM, and capillary tube formation, occurred in the in vitro system using human microvascular endothelial cells.

Because, in the absence of added angiogenesis factor, no HDMEC sprouts emanated from the EC-bead surface into the surrounding fibrin gel (Figure 2a), the degree of HDMEC angiogenesis response to increasing concentrations of angiogenesis factor could be easily quantified by determination of the number of beads with sprout formation. In addition the length of individual sprouts/tubes, and the degree of network formation can be

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easily quantified. VEGF (0 ng/ml - 100 ng/ml) induced a positive dose-response of sprout angiogenesis at 48 hr (Figure 4). Moreover, in the presence of 100 ng/ml VEGF, a capillary-like network formed by 7 days (Figure 5a).

5 When observed at high magnification, these capillary-like structures appeared to be composed of linear arrays of multiple HDMEC (Figure 5b). Networks (Figure 5c) formed by branching and fusion of capillary tubes from adjacent beads (Figure 5d).

10

EXAMPLE III

Induction of HDMEC capillary sprout formation by VEGF, VEGF-C, bFGF and PDGF-BB

Considerable evidence demonstrates that VEGF, VEGF-
15 C, bFGF and PDGF-BB are involved in stimulating angiogenesis in vivo (Oh et al. 1997; Friedlander et al. 1995; Risau et al. 1992). However, their direct angiogenic effect on human microvascular endothelial cells has not yet been well defined in vitro. The effect
20 of each of these factors on capillary sprout formation by HDMEC in fibrin gel was determined at 48 hr and quantified by calculating the percent of beads with sprouts. VEGF-C, a member of the VEGF family, recently has been determined to be an important growth factor for
25 the lymphatic vascular system that has potent angiogenic effects in vivo (Oh et al. 1997). Similar to VEGF, VEGF-C dose-dependently induced sprout angiogenesis of HDMEC in fibrin (Figure 6). However, its stimulatory effect was markedly less than that of VEGF, with maximal
30 response at 100 ng/ml only about 50% of the response to VEGF. bFGF strongly induced sprout angiogenesis of HDMEC in fibrin (Figure 7), with maximal response at 30 ng/ml comparable to that of VEGF at 100 ng/ml. The angiogenic stimulatory effect declined at higher concentrations of

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bFGF (50-100 ng/ml). Although PDGF-BB has been reported to be involved in angiogenesis in vivo (Risau et al. 1992), it did not significantly induce sprout angiogenesis of HDMEC in fibrin at concentrations ranging from 10 to 100 ng/ml (Figure 7). Thus, VEGF, VEGF-C, bFGF and PDGF-BB differentially regulated sprout angiogenesis of HDMEC in fibrin in vitro. VEGF and bFGF were strong direct angiogenic factors for HDMEC in fibrin.

10

EXAMPLE IV

Differential regulation of angiogenic response of HDMEC by fibrin and collagen gel

The process of angiogenesis is a dynamic interaction of microvascular endothelial cells with angiogenesis factors and the surrounding ECM milieu (Madri et al. 1996). To understand the relationship between angiogenesis and different ECM components during granulation tissue formation of wound repair, tissue specimens from 5, 7 and 10 day porcine wounds were analyzed. Previously other investigators have shown that 5 day wounds are mainly composed of a fibrin-rich provisional matrix, whereas 7 day wounds have a substantial organized collagen fiber network, and 10 days wounds have developed a compacted contracted collagen scar (Clark et al. 1982a and 1982b; Welch et al. 1990; Clark et al. 1995). Staining of 5, 7, and 10 day wound specimens with Masson trichrome (Figure 8a, c, e) and with anti-laminin, as a marker for blood vessels (Figure 8b,d,f), revealed that the fibrin-rich early granulation tissue in 5 day wounds is filled with newly formed vessels (Figure 8a,b). These neovessels consistently stained weakly for laminin, most likely as a result of blood vessel immaturity. Such weak staining for laminin

in immature blood vessels was previously observed in the microvasculature of human fetal skin (Tonnesen et al. 1985). By 7 days, the maturing blood vessels form an organized vertical array as collagen accumulates in the wound ECM (Figure 8c,d). At 10 days, as the collagen bundles thicken to produce scar, many blood vessels are regressing (Figure 8e,f). Thus, during wound repair in vivo, the angiogenic neovessels in early granulation tissue mature and then regress as fibrin is replaced by collagen in the wound space (Figure 9).

To determine whether fibrin and collagen differentially regulated the angiogenic response of microvascular endothelial cells to angiogenic factors, the angiogenic response of HDMEC in 3-dimensional fibrin and collagen gels in response to VEGF was compared, since VEGF has been reported to be abundantly present in early wounds, but not in normal skin (Brown et al. 1992). EC-beads were embedded in fibrin gel or in collagen gel, with or without the addition of VEGF for 48 hr. In the absence of VEGF, HDMEC remained on the surface of EC-beads and did not invade either fibrin gel (Figure 10a) or collagen gel (Figure 10b). In the presence of VEGF in fibrin gel, HDMEC formed capillary-like sprouts from the surface of EC-beads and invaded and migrated into the surrounding fibrin (Figure 10c). In contrast, when VEGF was added to collagen gel, HDMEC invaded and migrated into the surrounding collagen as individual cells, but did not form sprouts (Figure 10d).

To confirm that in this 3-dimensional ECM system, invasive angiogenic sprout formation induced by VEGF depended on the presence of fibrin, EC-beads were embedded in collagen admixed with fibrin and the angiogenic response to that in either pure fibrin or pure collagen gel was compared. In collagen gel without

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fibrin (Figure 11a), VEGF induced individual cell invasion and migration, as previously observed. With addition of fibrin to collagen gel (Figure 11b, 17% fibrin, and Figure 11c, 29% fibrin), sprout formation
5 occurred together with individual cell invasion and migration. In pure fibrin (Figure 11d), VEGF induced sprout angiogenesis. Thus the presence of fibrin appeared to be essential for HDMEC sprout angiogenesis induced by VEGF.

10

EXAMPLE V

Promotion of Angiogenesis and Vasculogenesis Using Cultured Endothelial Cells

Cultured endothelial cells grow well as aggregates.
15 When in the proper environment, these aggregates can form capillary-like tubes. This in vitro phenomenon simulates vasculogenesis as observed during fetal development. Vasculogenesis in tissues can be promoted by implanting endothelial cell aggregates, and angiogenesis in tissues
20 can be promoted by implanting endothelial cells on microcarriers.

An endothelial cell microcarrier system using dextran beads is described in the Materials and Methods, and Examples II-IV. Capillary-like tubes sprout from
25 these beads when they are placed in the proper environment. No capillary sprouts are observed when beads loaded with endothelial cells are placed in either fibrin gels or type 1 collagen gels without an angiogenesis stimulator. When vascular endothelial cell
30 growth factor (VEGF) is present in the fibrin gel, capillary tubes sprout from the beads. In contrast, no sprouts are observed when VEGF is added to collagen gels. Instead, single endothelial cells migrate off of the beads into the surrounding 3-dimensional collagen matrix.

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In accordance with the subject invention, endothelial cell aggregates or endothelial cells on microcarriers are added to wounds or other tissues that need capillary bed enhancement. The endothelial cell aggregates or endothelial cells on microcarriers can also be added to artificial tissue constructs to promote vasculogenesis or angiogenesis, respectively, within these constructs. Examples of artificial skin constructs includes systems produced by Organogenesis (Boston, MA),
5
10 Ortec (NY, NY), and Integra Life Sciences (Princeton, NJ).

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various
15 modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which
20 follow.

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What Is Claimed Is:

1 1. A cellular matrix system comprising:
2 cells of a selected cell type disposed on
3 microcarrier particles; and
4 an in vitro defined matrix having the microcarrier
5 particles disposed therein, wherein the in vitro matrix
6 simulates a natural environment in which the cells
7 naturally reside.

1 2. The cellular matrix system of claim 1 wherein
2 the cells of a selected cell type are endothelial cells.

1 3. The cellular matrix system of claim 2 wherein
2 the endothelial cells are cultured human dermal
3 microvascular endothelial cells.

1 4. The cellular matrix system of claim 2 wherein
2 the in vitro defined matrix is a fibrin matrix.

1 5. The cellular matrix system of claim 2 wherein
2 the in vitro defined matrix is a collagen matrix.

1 6. The cellular matrix system of claim 1 wherein
2 the microcarrier particles are pre-conditioned.

1 7. The cellular matrix system of claim 1 wherein
2 the microcarrier particles are bioresorbable.

1 8. The cellular matrix system of claim 1 further
2 comprising cells of a different selected cell type
3 overlaid by or admixed with the in vitro defined matrix
4 having the microcarrier particles disposed therein.

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1 9. The cellular matrix system of claim 8 wherein
2 the cells of a different selected cell type are tumor
3 cells.

1 10. The cellular matrix system of claim 8 wherein
2 the cells of a different selected cell type are provided
3 as a layer of cells, and wherein the in vitro defined
4 matrix overlies the layer.

1 11. The cellular matrix system of claim 8 wherein
2 the cells of a different selected cell type are provided
3 as a droplet or aggregate of cells, and wherein the in
4 vitro defined matrix overlies or is admixed with the
5 droplet or aggregate.

1 12. A cellular angiogenesis system comprising:
2 endothelial cells disposed on microcarrier
3 particles; and
4 an in vitro defined fibrin matrix having the
5 microcarrier particles disposed therein.

1 13. A method of determining the effect on cellular
2 migration/angiogenesis of a compound or effector cell of
3 interest, the method comprising:
4 providing cells of a selected cell type on
5 microcarrier particles which are disposed in an in vitro
6 defined matrix, wherein the in vitro defined matrix
7 simulates a natural environment in which the cells
8 naturally reside;
9 determining migration/angiogenesis of the cells into
10 the in vitro defined matrix;
11 including a compound or effector cell of interest in
12 the in vitro defined matrix;

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13 redetermining migration/angiogenesis of the cells
14 into the in vitro defined matrix which includes the
15 compound or effector cell of interest; and
16 determining the effect on cellular
17 migration/angiogenesis of the compound or effector cell
18 of interest.

1 14. The method of claim 13 wherein the cells of a
2 selected cell type are endothelial cells.

1 15. The method of claim 14 wherein the endothelial
2 cells are cultured human dermal microvascular endothelial
3 cells.

1 16. The method of claim 14 wherein the in vitro
2 matrix is a fibrin matrix.

1 17. The method of claim 14 wherein the in vitro
2 defined matrix is a collagen matrix.

1 18. The method of claim 13 wherein the microcarrier
2 particles are pre-conditioned.

1 19. The method of claim 13 wherein the microcarrier
2 particles are bioresorbable.

1 20. The method of claim 13 further comprising
2 overlaying or admixing with the in vitro defined matrix
3 having the microcarrier particles disposed therein cells
4 of a different selected cell type, and detecting
5 migration/angiogenesis of the cells of the selected cell
6 type into the cells of the different selected cell type.

1 21. The method of claim 20 wherein the cells of the
2 different selected cell type are tumor cells.

1 22. The method of claim 20 wherein the cells of the
2 different selected cell type are provided as a layer of
3 cells, and wherein the in vitro defined matrix overlies
4 the layer.

1 23. The method of claim 20 wherein the cells of the
2 different selected cell type are provided as a droplet or
3 aggregate, and wherein the in vitro defined matrix
4 overlies or is admixed with the droplet or aggregate.

1 24. The method of claim 13 wherein the compound or
2 effector cell of interest stimulates
3 migration/angiogenesis.

1 25. A method of determining the effect on
2 angiogenesis of a compound or effector cell of interest,
3 the method comprising:
4 providing endothelial cells on microcarrier
5 particles which are disposed in an in vitro defined
6 fibrin matrix;
7 determining angiogenesis of the cells into the in
8 vitro defined fibrin matrix;
9 including a compound or effector cell of interest in
10 the in vitro defined fibrin matrix;
11 redetermining angiogenesis of the cells into the in
12 vitro defined fibrin matrix which includes the compound
13 or effector cell of interest; and
14 determining the effect on angiogenesis of the
15 compound or effector cell of interest.

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1 26. A method of promoting angiogenesis in a tissue,
2 the method comprising:
3 selecting a tissue in which angiogenesis is desired;
4 and
5 introducing cultured endothelial cells into the
6 tissue to promote angiogenesis in the tissue.

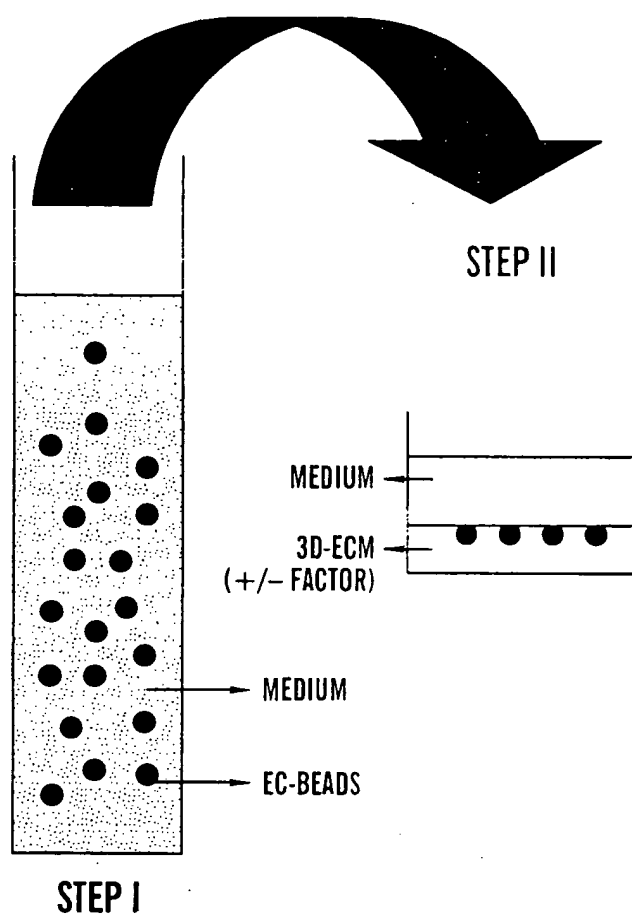
1 27. The method of claim 26 wherein the tissue is a
2 wound.

1 28. The method of claim 26 wherein the tissue is an
2 artificial tissue.

1 29. The method of claim 26 wherein the cultured
2 endothelial cells are introduced as an endothelial cell
3 aggregate.

1 30. The method of claim 26 wherein the cultured
2 endothelial cells are introduced as endothelial cells
3 disposed on microcarrier particles.

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**FIG. 1**

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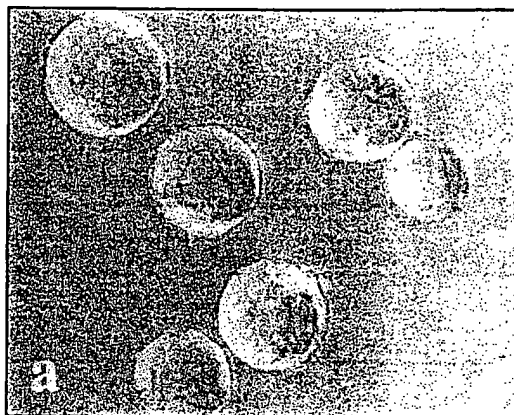


FIG. 2A

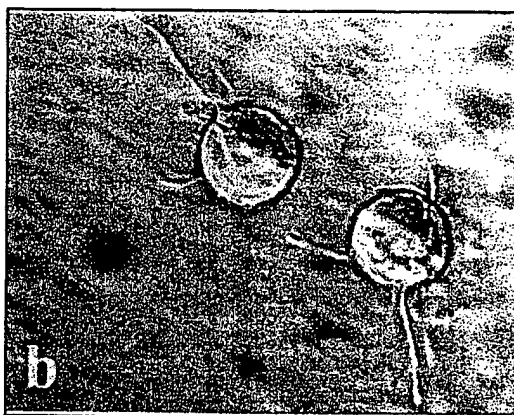


FIG. 2B

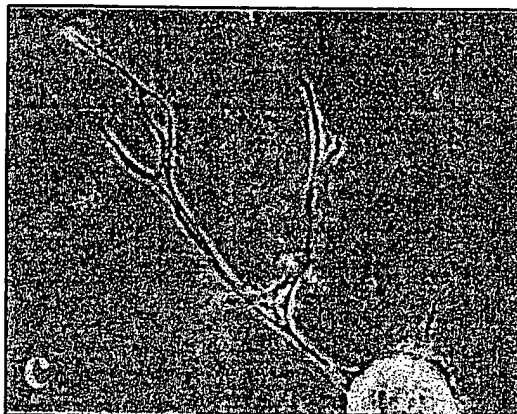


FIG. 2C

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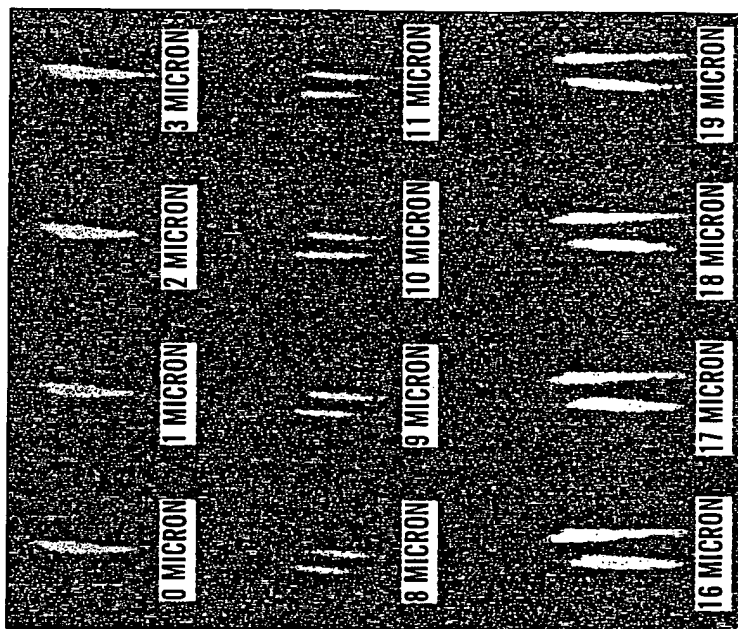
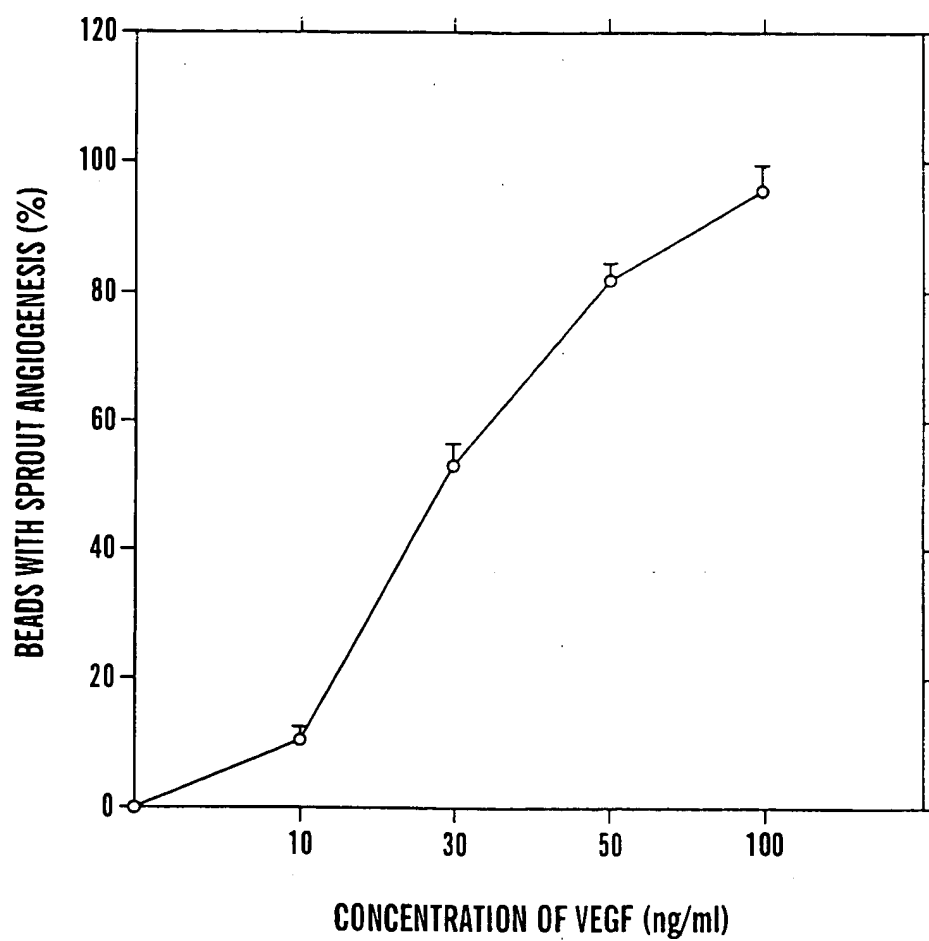


FIG. 3B



FIG. 3A

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**FIG. 4**

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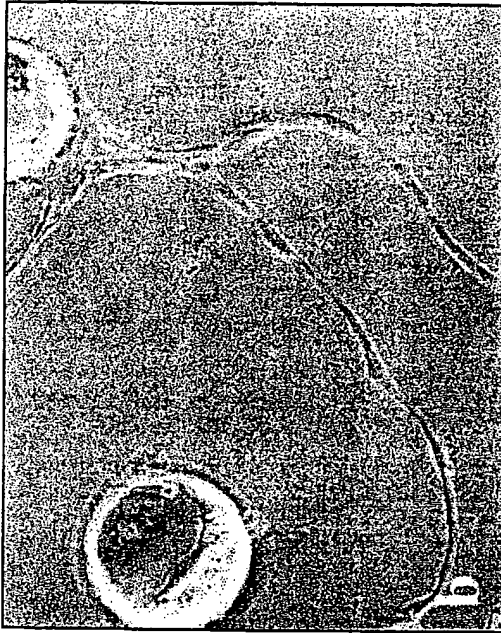


FIG. 5B

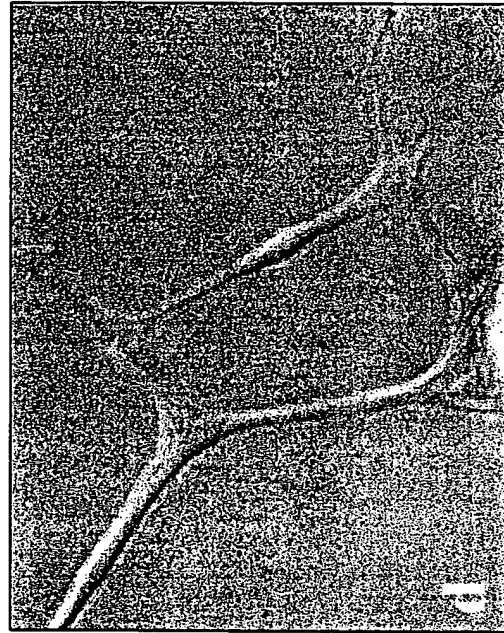


FIG. 5D

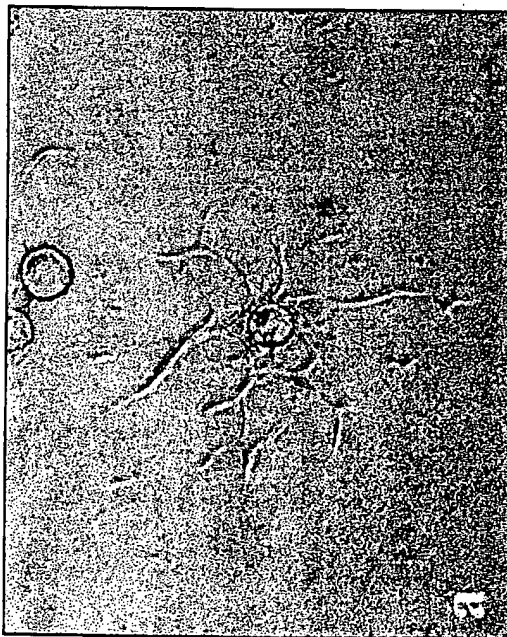


FIG. 5A

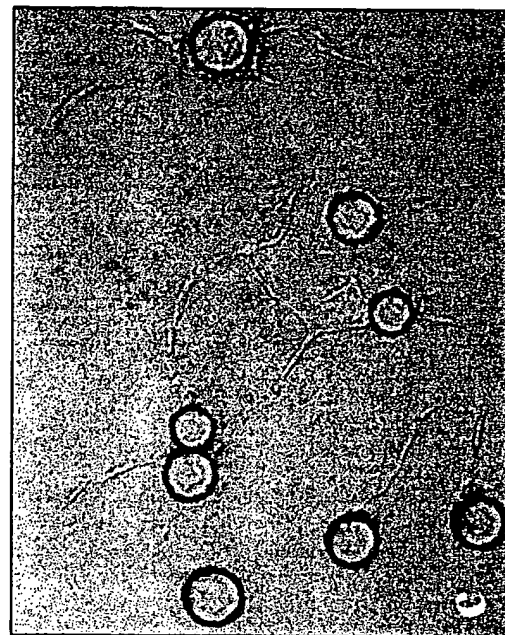
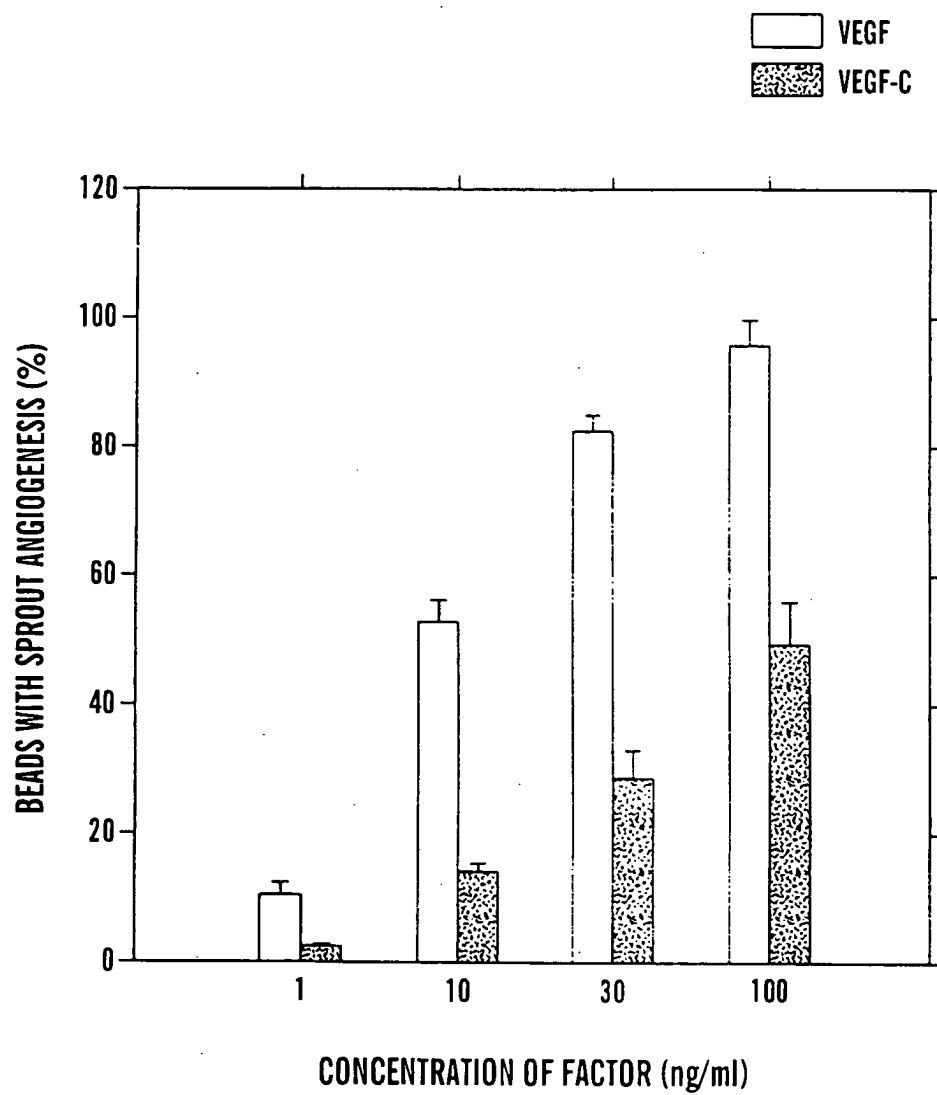
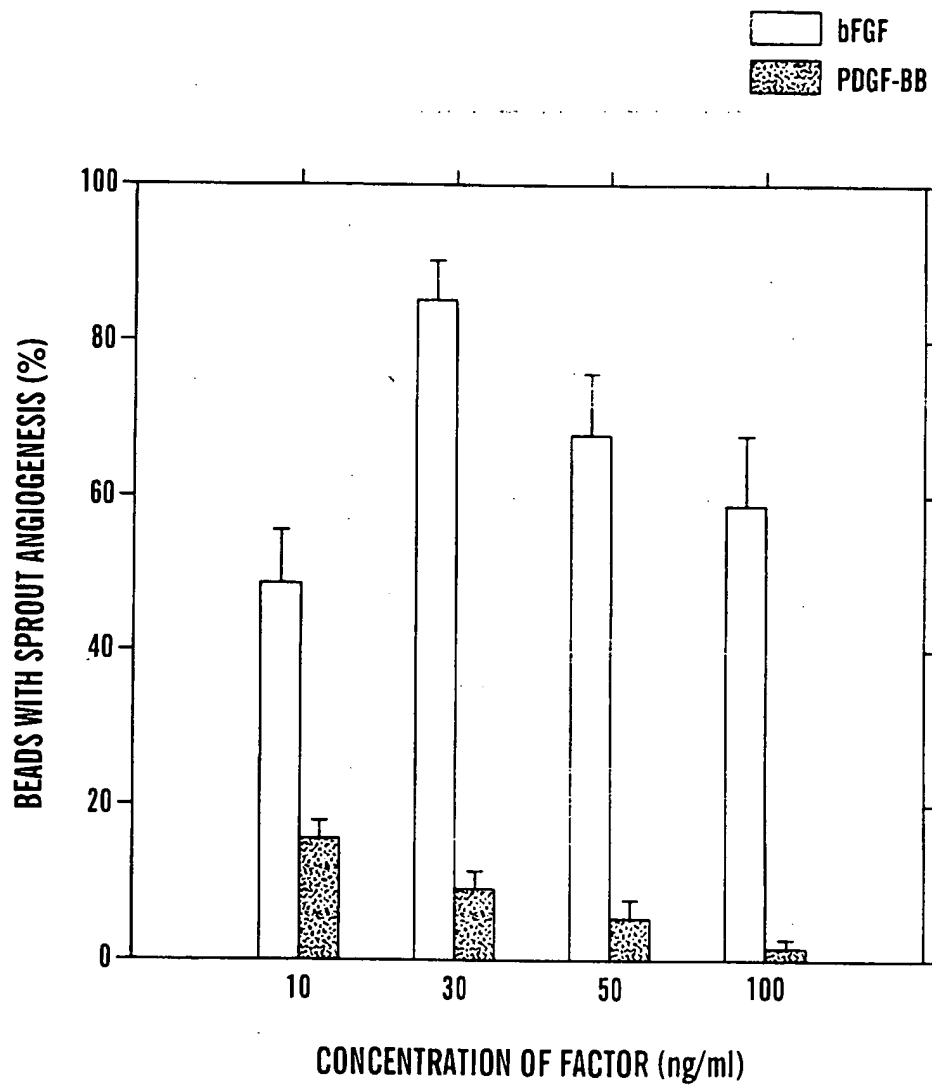


FIG. 5C

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**FIG. 6**

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**FIG. 7**

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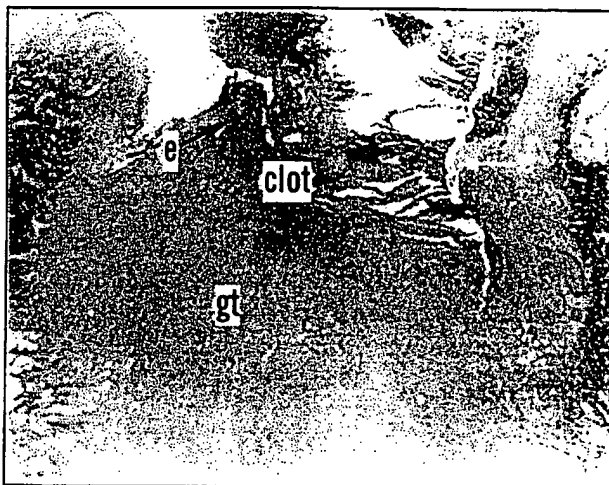


FIG. 8A

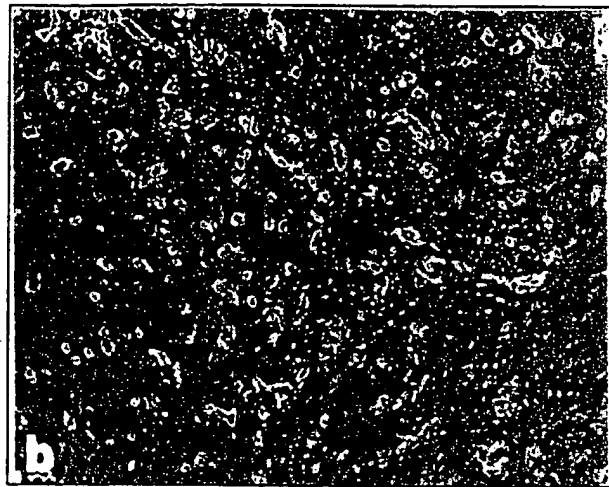


FIG. 8B

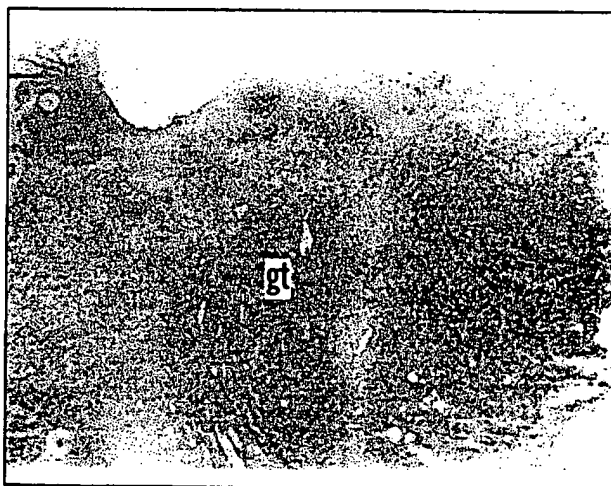


FIG. 8C

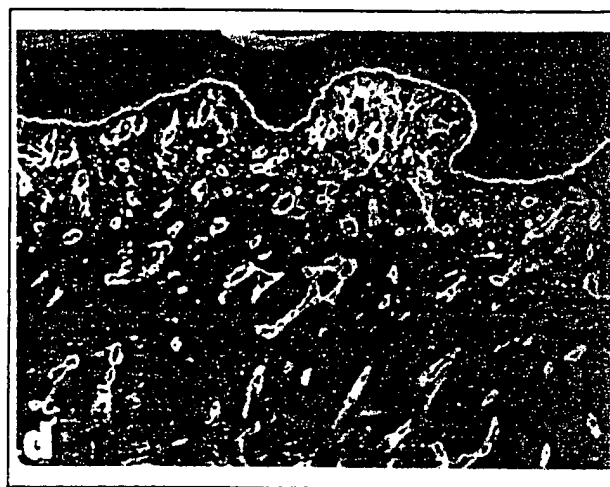


FIG. 8D

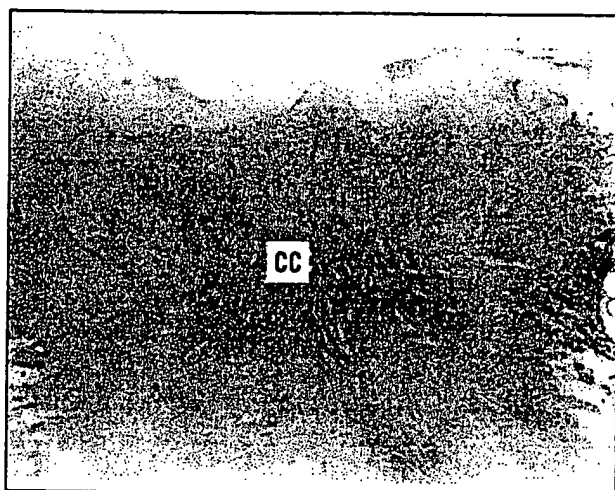


FIG. 8E

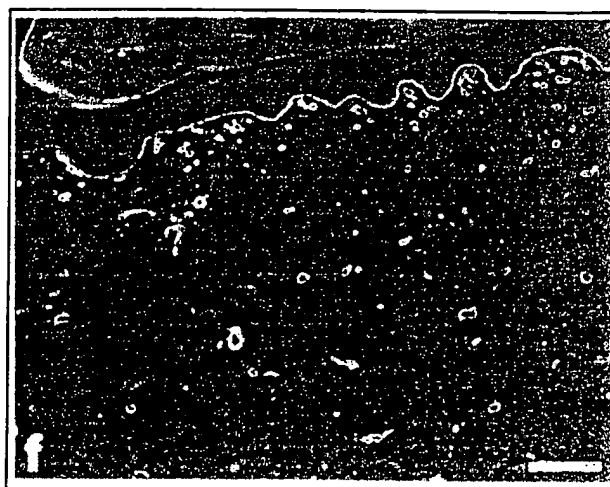


FIG. 8F

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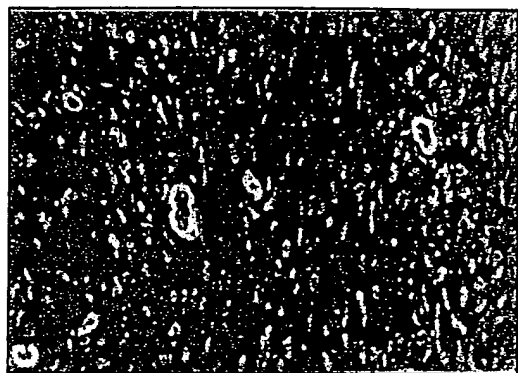


FIG. 9C

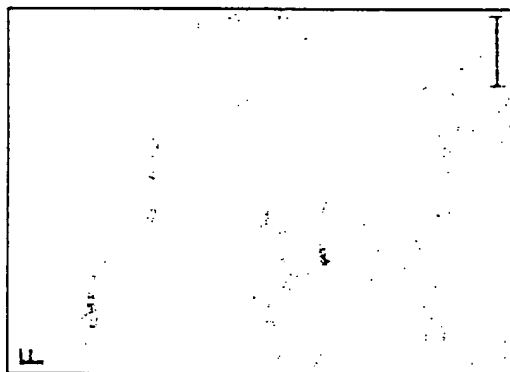


FIG. 9F



FIG. 9B

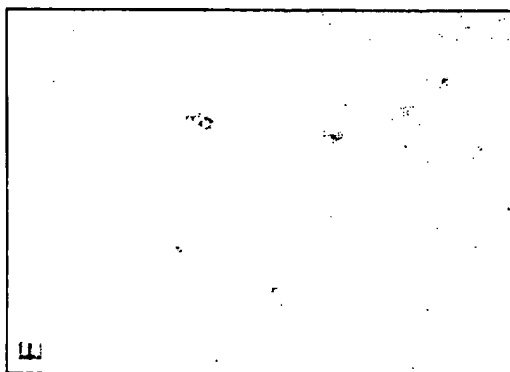


FIG. 9E

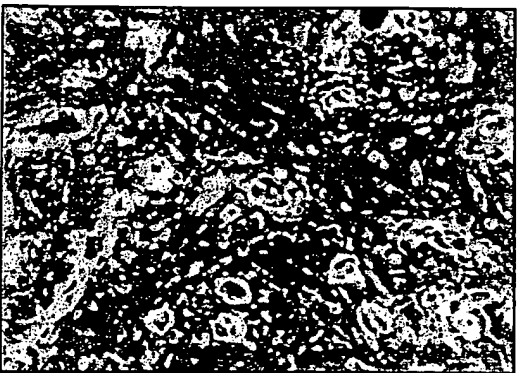


FIG. 9A

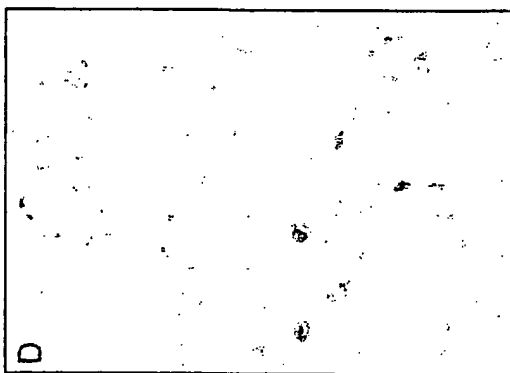


FIG. 9D

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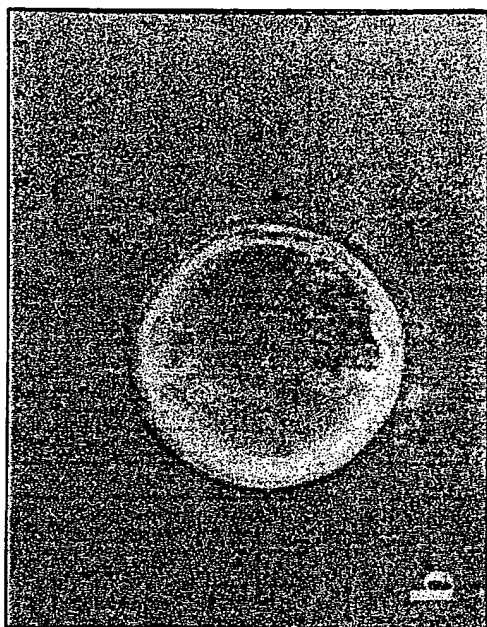


FIG. 10B

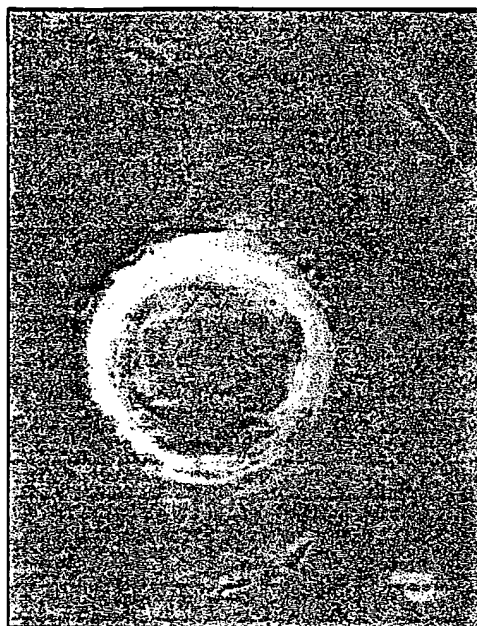


FIG. 10D

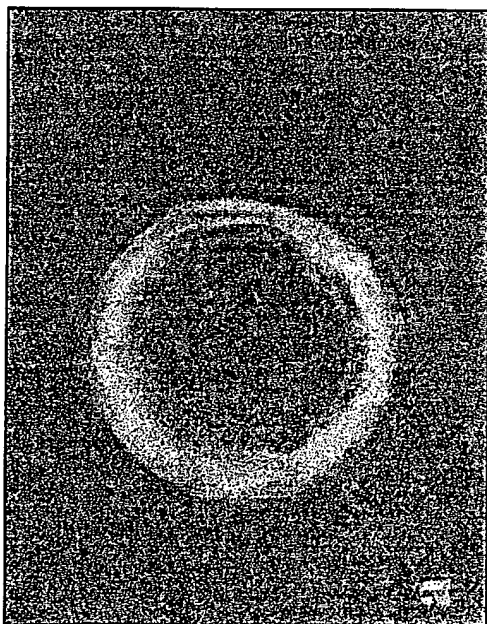


FIG. 10A



FIG. 10C

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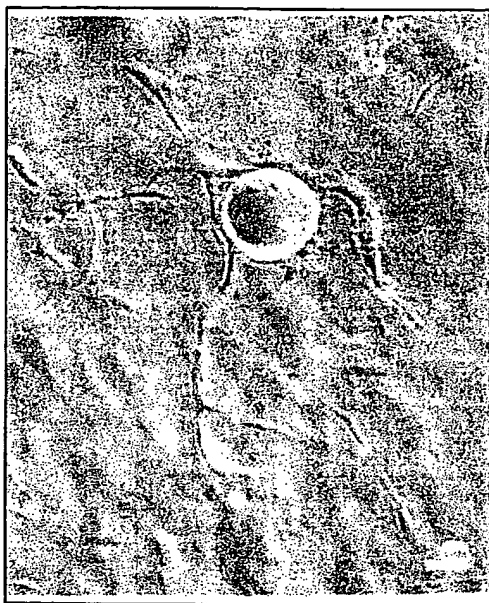


FIG. 11B

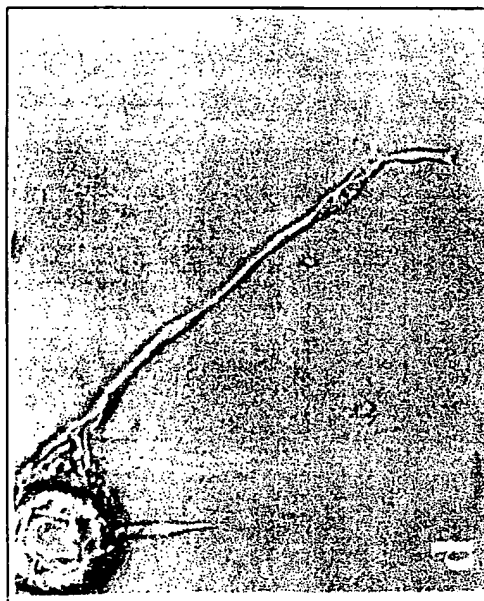


FIG. 11D



FIG. 11A

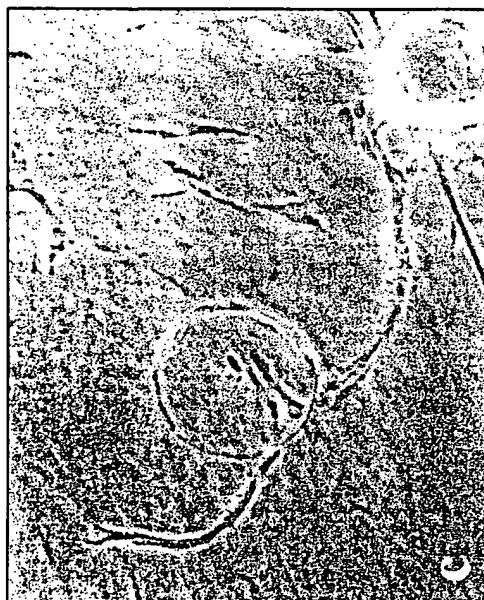


FIG. 11C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/10091

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61L27/22 A61L27/24 A61L27/38 A61L27/60 C12N11/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 10217 A (VITAPHORE WOUND HEALING INC) 25 June 1992 (1992-06-25) abstract; claims	1-3,5-7, 13-15, 17-20, 22,24, 26,27,30
X	US 5 804 178 A (JOHNSON LYNT ET AL) 8 September 1998 (1998-09-08) abstract column 5, line 61; claims	1,2,5-8, 10,13, 14, 17-20, 22,24, 26-28,30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

14 July 2000

Date of mailing of the international search report

04. 09. 2000

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Authorized officer

Krenn

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/10091

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EP 0 518 389 A (UNIV JEFFERSON) 16 December 1992 (1992-12-16)</p> <p>abstract page 14, line 8 - line 26; claims -----</p>	<p>1-7, 12-20, 22, 24-26, 28,30</p>

INTERNATIONAL SEARCH REPORT

Internat. application No.

SAE 280257

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 26-30 (please see remark)
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 26-30 are directed to a therapeutic method of treatment of (diagnostic method practised on) the human/animal body, the search report has been carried out and based on the alleged effects of the compound/
composition (see PCT-Rule 39.1 (iv)).
2. ☐ Claims Nos.: composition (see PCT-Rule 39.1 (iv)).
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/10091

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9210217 A	25-06-1992	AU 8952691 A EP 0560865 A JP 6503735 T ZA 9109594 A	08-07-1992 22-09-1993 28-04-1994 26-08-1992
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EP 0518389 A	16-12-1992	US 4820626 A AT 113850 T AU 590573 B AU 5830586 A BR 8602659 A CA 1293700 A DE 3650134 D DE 3650134 T DE 206025 T EP 0206025 A ES 555739 D ES 8900149 A IL 78950 A JP 2686930 B JP 62049857 A MX 165125 B US 5441539 A US 5035708 A US 5194373 A US 5230693 A US 5372945 A US 5312380 A US 5628781 A ZA 8603958 A ES 2004281 A US 5131907 A	11-04-1989 15-11-1994 09-11-1989 11-12-1986 03-02-1987 31-12-1991 15-12-1994 22-06-1995 15-10-1987 30-12-1986 01-03-1989 01-05-1989 15-12-1991 08-12-1997 04-03-1987 28-10-1992 15-08-1995 30-07-1991 16-03-1993 27-07-1993 13-12-1994 17-05-1994 13-05-1997 28-01-1987 16-12-1988 21-07-1992